

**An investigation into the effects of  
ectopic expression of transporter  
genes during seed development of  
*Pisum sativum* L.**

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of the requirements for the Degree of  
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By

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### *“LORD IS MY SHEPHERD, I SHALL NOT WANT”*

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## Abstract

Increases in yield of food plants are urgently required. Identification of new breeding targets for genetic improvement is critical, and for which transgenic plants can provide proof-of-concept. Flower number, fruit set and seed development are crucial components of final yield in legumes and import of sucrose together with amino acids and amides largely accounts for the biomass gain and quality of the seed. Cytokinins have been implicated at all three stages of reproductive development. Besides an effect on cell division and sink establishment, the cytokinins have been implicated in the regulation of source-sink relationships.

Previously developed double transgenic lines expressing both a *PsAAP* (an amino acid permease) and a *PsSUT* (a sucrose transporter) had increased seed yield due to a significant increase in seed number compared to their corresponding wild-type cultivar. This study assessed the expression of *PsSUT*, *PsAAP*, cytokinin, cell wall invertase, and SWEET gene family members during seed development in two cultivars of *Pisum sativum* L. Subsequently, young pods, seeds, seed coats and leaves from wild type and transgenic plants over-expressing the *PsAAP* and *PsSUT* genes were analysed to identify what effect the transgenes had had on influencing gene expression in the source and sink tissues.

The genes related to cytokinin homeostasis, nutrient transport and metabolism were isolated and assessed with polymerase chain reaction (PCR) and real time-quantitative PCR (RT-qPCR) analysis. Differences were seen in the spatial and temporal expression of particular gene family members between the wild-types and their transgenic lines.

In conclusion, it is evident from the data that the yield increase could be the result of enhancing both sucrose and amino acid import to the seed coat and thence to the seed, along with enhanced cytokinin and cell wall invertase activity during early stages of development. It is also evident from the data that the double transgenes had a much wider effect on the whole plant as evidenced by the enhanced gene expression in the younger source leaves.

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## Abbreviations

AAP	amino acid permease
ADP	adenosine diphosphate
AHK	<i>Arabidopsis</i> histidine kinase
AMP	adenosine monophosphate
BLAST	basic local alignment tool
bp	base pairs
cDNA	complementary DNA
CKX	cytokinin oxidase/dehydrogenase
cZ	<i>cis</i> -zeatin
DAF	day after flowering
DMAPP	dimethylallyl diphosphate
DW	dry weight
DZ	dihydrozeatin
FAD	flavine adenine dinucleotide
FW	fresh weight
GUS	$\beta$ -glucoronidase
IAA	indole-3-acetic acid
iP	isopentenyladenine

iPA	isopentenyladenosine
IPT	isopentenyltransferase
LOG	The Lonely Guy
mRNA	messenger RNA
MVA	mevalonate
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
RWC	relative water content
SUC	sucrose carrier
SUF	sucrose facilitator
SUT	sucrose transporter
SWEET	sugars will be eventually exported transporters
tRNA	transfer RNA
tZ	<i>trans</i> -zeatin
Z	zeatin
ZR	zeatin riboside



# Chapter 1

## Introduction

### 1.1 Background

New Zealand seed and grain production occupies about 150,000 hectares and the seed industry has an estimated value of \$370 million (<http://www.nzgsta.co.nz>). The “Advanced Seed Production Systems” contract, which funded the research in this thesis was part of a FRST funded program, which aimed to enhance seed quality and quantity for the producers of the seed. It also aimed to overcome the financial uncertainty with indeterminate plants by providing technology-led seed production systems for consistent high seed yields and assured seed quality. The export of seed has long been a feature of the New Zealand seed industry (<http://www.nzgsta.co.nz/>).

Canterbury Seeds is one of the largest producers of *Pisum sativum* L. (pea) seeds in New Zealand for vining/freezing, and develops new pea varieties through an exclusive breeding program. Their focus includes pod type, pea size, flavour and maturity, as well as robustness of the plant to climatic and disease pressure. In New Zealand, peas are grown for fresh, frozen and canned human food and are exported as peas for sowing, and as dried peas and frozen peas for consumption (<http://seedquest.com>).

Legumes are a particularly important source of proteins, vitamins and minerals. Among these, peas are at the highest level as producers of protein (Johnson and Lay 1974). Dry pea is nutritionally rich, containing 18 to 33% protein, 4 to 7% fiber, and 37 to 49% starch (Bastianelli et al., 1998) and is consumed as a staple protein source in many developing countries (McPhee, Genome Mapping and Molecular Breeding in Plants, Vol.3). The nutritional value of immature fresh peas and mature dry peas for both human and animal consumption has fostered sustained production. Mature dry seed is used predominantly as an animal feed but is also a component of soups for human consumption and can be processed industrially for starch or protein preparations (<http://www.agriculture.gov.sk.ca>). Fresh pea production for the canning and freezing industry includes the harvest of whole pods for use in

oriental preparations and snap peas, which can be eaten fresh or frozen

(<http://www.nzgsta.co.nz/>).

Breeding objectives for pea vary depending on production region and end use. Quality of fresh peas is highly refined, and genetics controlling specific traits are controlled through proprietary means. The challenge to plant breeders is to develop crop varieties that are both more productive and more nutritious than the current varieties

(<http://agrobiol.sggw.waw.pl/cbcs>). Quality attributes of peas grown for dry seed harvest have focused primarily on the visual appearance of the seed, i.e., uniformity of seed colour and intensity as well as shape (<http://maxa.maf.govt.nz/>). Greater attention is being given to the chemical composition of the pea due to its importance in animal feed rations

(<http://www.hortnz.co.nz/>). Major targets for improvement in pea breeding programmes include seed yield and seed size (quantity), and flavour, sweetness and protein content (quality).

It has been suggested that improvement in protein content can be achieved by influencing transporter genes which play a significant role in transporting sucrose and amino acids into seeds (Tegeder et al., 2000). The expression and localisation of these genes support a function of the transporters in phloem loading for amino acids and sucrose, translocation to sinks, as well as in seed loading for development and storage of protein and starch (Tegeder et al., 2007).

The seed represents the unit of reproduction of flowering plants. Seed development begins with double fertilisation, the products of which are a diploid zygote and a triploid primary endosperm cell. The latter develops into a storage endosperm tissue that is structurally adapted to ensure efficient translocation of nutrients to the developing embryo. However, the progress of development differs between monocots and eudicots: in monocots the endosperm constitutes the major part of the mature seed; in many eudicots the endosperm grows rapidly initially, but is eventually consumed by the cotyledons of the developing embryo, which occupies most of the mature seed (Sundaresan, 2005). The seed coat arises from the maternal integument tissue, whereas both the triploid endosperm and diploid embryo are filial tissue. The size of the seed is primarily associated with the initial growth of the endosperm and not with the later growth of the embryo (Sundaresan, 2005; Mizutami et al., 2010).

The cytokinins have been implicated in seed development in both monocots and dicots (Song et al., 2015), through their effect on cell division and ultimately seed size. Additionally, they have been shown to be limiting to seed number (Ashikari et al., 2005b, Bartrina et al., 2011). The focus in this project was on the genes involved in establishing both quality and quantity of the harvested pea seeds as well as the interaction between transporter genes and cytokinin biosynthesis and metabolism genes.

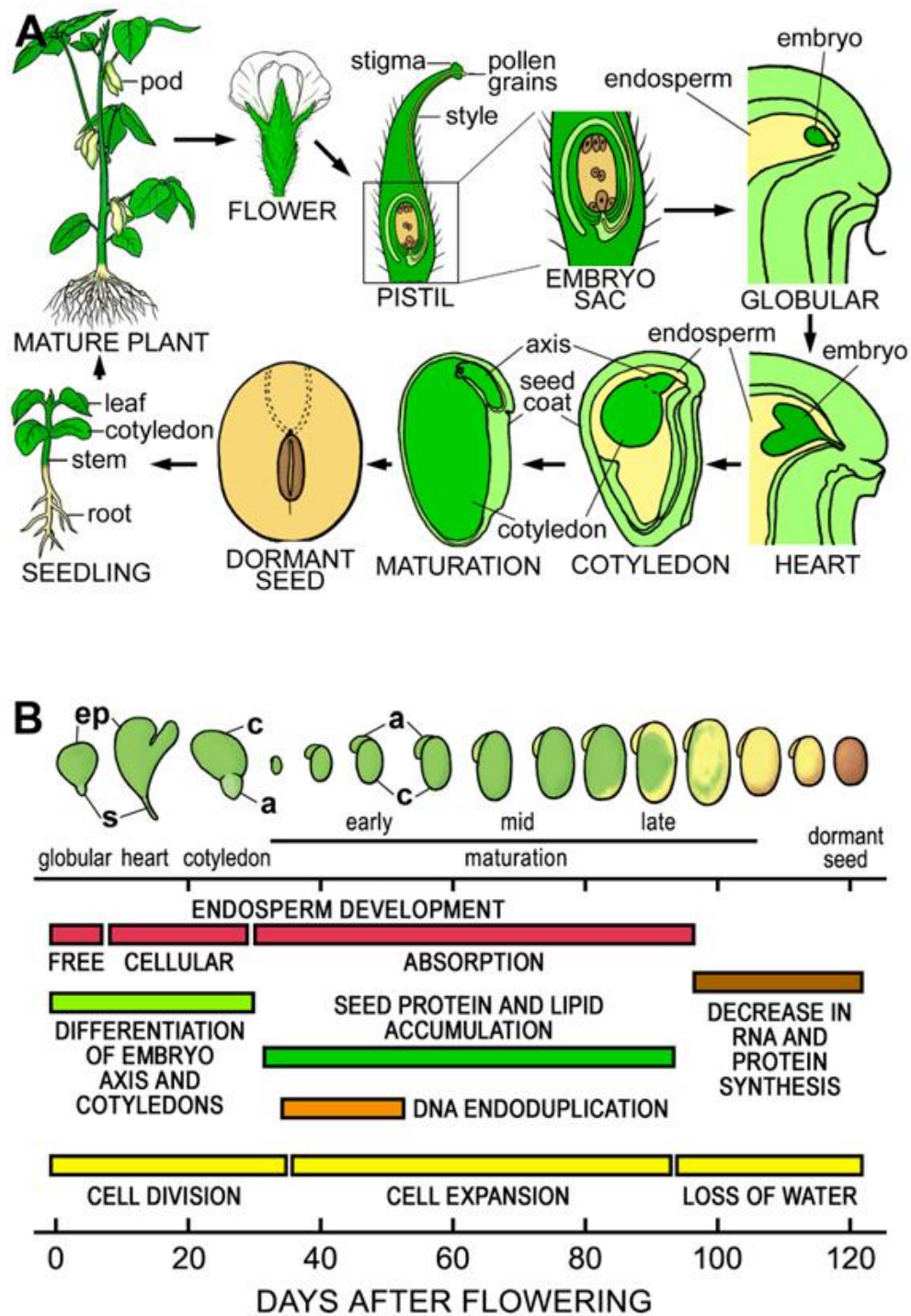
## **1.2 Peas**

*P. sativum* L. is one of the world's oldest domesticated crops (Zohary, 2000). Pea was the original model plant used in Mendel's work (1866), on the laws of inheritance, making it the foundation of modern plant genetics. Pea belongs to the Leguminosae family, which has an important ecological role because it contributes to the development of low-input farming systems by fixing atmospheric nitrogen and it serves as a break crop which further minimizes the need for external inputs (Lewis, 2005). Pea is a diploid plant with 14 chromosomes ( $n=7$ ) and it has been commonly used as a model plant for studies in plant physiology and biotechnology due to its well defined genetics. Due to their large size and morphology, seeds of *P. sativum* offer excellent models to study how seed metabolism is connected to growth and development and how transporters control biosynthetic pathways (Borisjuk et al., 2003).

## **1.3 Legume seed development**

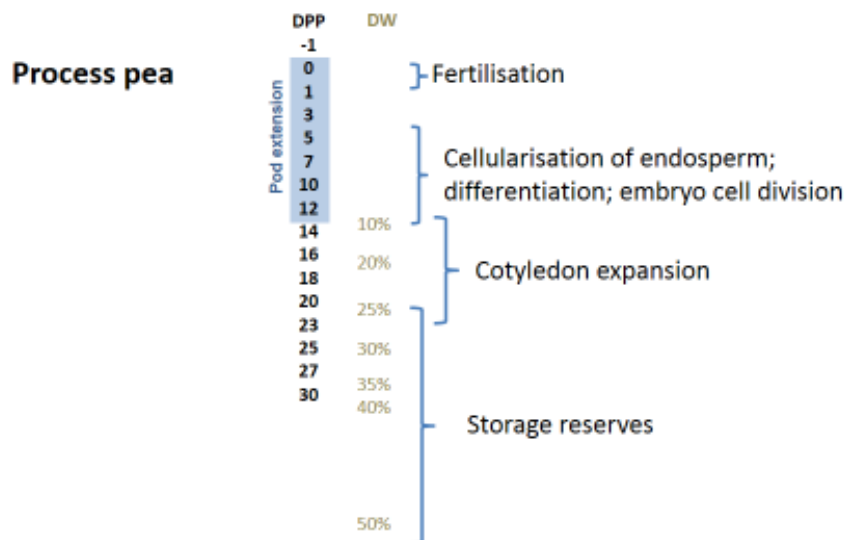
Developing legume seeds are complex structures, containing embryo and other tissues including seed coat, endosperm and suspensor (Fig 1.1) (Weber et al., 1997). Seed development is often considered to occur in two distinct phases: the first during which endosperm development, cell divisions, and embryo and cotyledon differentiation occurs, is referred to as the morphogenesis phase. In legume development this is sometimes referred to as the pre-storage phase (Weber, 2005). The second phase, referred to as maturation, includes embryo growth by cell expansion, the absorption of the endosperm by the embryo, and dry

matter accumulation (Locasio et al., 2014).



**Figure 1.1: Image depicting the life cycle of soybean, from flower to seed development.**  
Copied from Plant Physiology 144(2): 562-574. (Brandon H. Le, 2007)

Studies suggest that legume seeds develop in three rapid phases of growth separated by two lag phases. The first growth stage is limited to endosperm and seed coat, while the second phase is associated with embryo growth by cell division. A second lag phase precedes the third growth period, which is the maturation phase characterised by cell expansion and accumulation of storage products (Wang, 1993). During development, seed coat, endosperm and embryo interact physically (Weber et al., 1996). The whole process is genetically programmed and under metabolic control (Weber, 2005). A generalised diagram of different stages of pea seed development is shown in Fig 1.2.



**Figure 1.2: Generalised diagram showing different stages of seed development in process pea.** DAP means days after pollination, DW means dry weight (Drawn by Jan Grant and Paula Jameson).

In crop seeds, the differentiation phase is of special interest because during this stage, the accumulation of storage products is initiated by a regulatory network. It is suggested that metabolite and hormone responsive pathways are involved in this (Wobus and Weber, 1999). Evidence shows that in pea seeds the general pattern of seed development is determined by

the maternal parent, the seed coat, but that the final seed size is correlated with the volume of endosperm (De Jong, 1997). In *V. faba* and pea, young seed coats are transient storage organs, which accumulate starch and proteins before storage activity starts in the embryo (Déjardin et al., 1997, Heim et al., 1993).

In plants, sucrose is the major transported form of carbohydrate. When seeds are maturing, this sucrose is then converted to its storage form, starch (KÜHN et al., 1996). Coats of developing seeds are functionally active in importing high fluxes of sucrose into the seed apoplast to meet the carbohydrate demand of the growing embryo (Patrick and Offler, 2001) and thus could be enriched in sucrose effluxers.

Many hormones and enzymes play a vital role during legume seed development. Seeds import sucrose, which must be hydrolysed before it is used for storage or in metabolic activity (Weber et al., 1996). Two key enzymes are involved to catalyze this cleavage, invertase and sucrose synthase which operate in a specific pathway. The invertase pathway is related to growth and cell expansion, whereas the sucrose synthase pathway is associated with storage product biosynthesis (Quick, 1996). Cell wall invertase (cwINV) is active in growing zones and expanding sink tissues and regulates assimilate unloading by increasing the sucrose concentration gradient (Weber et al., 1996). Studies showed that invertase activity is important for early seed development (Cheng, 1996).

During legume seed development, large amounts of protein are accumulated in the cotyledons. That means that the amino acids required for these storage proteins are imported through the funiculus and seed coat and finally taken up by the cotyledons (De Jong, 1997). It was shown that the amino acid transporters (AAP) were expressed in the storage parenchyma cells of pea (Tegeder et al., 2000). In *Arabidopsis*, *AtAAP1* expression was found exclusively in seeds, confirming a role in supplying amino acids to the seeds (Hirner et al., 1998).

During seed development, several sequential events have to be completed. They involve cell division and cell expansion, followed by accumulation of storage material. The accomplishment of these processes is associated with sink strength. Sink strength is determined by the size of the storage organ and its association with the number of cells that comprise the organ, and by the efficiency of unloading photosynthate from the conducting elements into the storage organ for the synthesis of the final storage products. Sink

establishment occurs at the early stage of seed development (Herbers and Sonnewald, 1998), which correlates with the highest level of cytokinins (Emery et al., 2000, Rijavec et al., 2009).

Cytokinins have a pivotal role in regulating seed development, which includes promoting cell division during embryogenesis, and directing the flow and accumulation of assimilates into the seed. Studies have shown that developing seeds are a rich source of cytokinins and seeds are capable of producing their own cytokinins (Letham and Palni, 1983, van Staden and Drewes, 1992, O'Keefe et al., 2011, Song et al., 2015). Furthermore, cytokinins supplied exogenously for developing seeds can influence seed development. It has been shown that injection of 6-benzylaminopurine into the pedicels of pea pods elevated the total protein content of developing seeds (Schroeder, 1984) and applying cytokinins to cereals just after anthesis enhanced grain setting and grain yield of wheat, oat and maize (Kamínek et al., 1997).

## **1.4 Cytokinins**

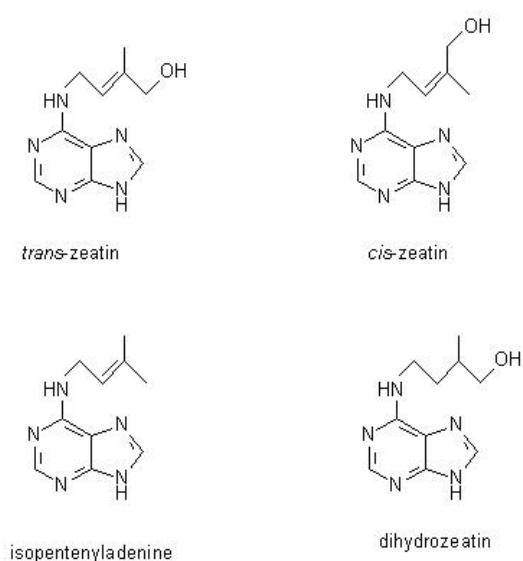
### **1.4.1 General role in plants**

Plant hormones play a vital role in seed development. Cytokinins are substances that induce cytokinesis in the presence of auxin. The term cytokinin generally refers to both naturally occurring and synthetic compounds with hormonal activities (Mok et al., 1992). The first characterised compound with cytokinin activity was kinetin which was isolated from autoclaved herring sperm DNA in 1955 (Letham, 1994). However, in 1963 the first naturally occurring cytokinin, zeatin was extracted and identified from immature maize caryopsis by Letham (1963). To date, many other naturally occurring cytokinin species have been identified. The cytokinins are a large and chemically diverse group of plant hormones and are involved in wide range of physiological and developmental processes (Mok et al., 1992). Cytokinins are now known to influence various aspects of plant growth and development, including cell proliferation and differentiation, nutritional signal transduction, fruit and seed development, delay of senescence, controlling the balance of roots and shoots, and apical dominance (Mok et al., 2000).

### 1.4.2 General forms - isoprenoid and aromatic

Cytokinins are *N*-6-substituted adenines. Naturally occurring cytokinins are adenine derivatives which carry either an isoprene-derived side chain or an aromatic side chain at the N<sup>6</sup>-terminus and are called isoprenoid or aromatic cytokinins respectively (Fig 1.3) (Sakakibara et al., 2006). Isoprenoid forms are predominant in higher plants and abundant in nature (Emery et al., 1998). Natural isoprenoid cytokinins can be seen as one of four basic forms, N<sup>6</sup>-isopentenyl adenine (iP), *cis*-zeatin (cZ), *trans*-zeatin (tZ), and dihydrozeatin (dZ). Each cytokinin nucleobase is distinguished by the characteristics of the side chain, namely the presence or the absence of a hydroxyl group at the end of the prenyl chain (tZ, cZ or iP) or the stereoisomeric position (cZ, tZ) or the saturation of the side chain (dZ) (Sakakibara et al., 2006).

#### Isoprenoid cytokinins – free bases



**Figure 1.3: Structures of the active cytokinin nucleobases occurring naturally.**

The relative biological activities of cytokinins were estimated by measuring their activity in bioassays and their affinity to cytokinin receptors (Inoue et al., 2001). *In vitro* and *in vivo* studies with *Arabidopsis* cytokinin receptors (AHK3 and AHK4/WOL/CRE1) showed that the receptors have much higher affinity for iP and tZ than for cZ and DZ (Inoue et al., 2001).



On the other hand, a cytokinin receptor from maize (ZmHK1) responded to cZ and tZ with similar sensitivity (Yonekura-Sakakibara et al., 2005), inferring that the relative activities of cytokinins might vary among plant species, but that the free bases are the active forms.

Both purine ring and side chain can be subjected to chemical modifications. Modification on the purine ring involves conjugation with sugar molecules, sugar phosphates and amino acids. Free bases are cytokinin species that most strongly bind to cytokinin receptors. Since these bindings are associated with physiological responses, these forms are biologically active. On the other hand, weakly bound cytokinin conjugates are biologically inactive. Among the well-defined conjugates, ribosides are the predominant forms found in the plant transport system, and hence they are considered as the transport forms (Takei et al., 2001, Kamada-Nobusada and Sakakibara, 2009).

Among glucosides, *O*-glucosides are reversibly deactivated forms of cytokinins and might serve for cytokinin storage, whereas 7- and 9-glucosides are presumed to be irreversibly inactivated end products of cytokinin metabolism, because the enzymes for the cleavage of sugars moieties from cytokinin 7- and 9-glucosides are not known in plants (Tomaz et al., 2010).

### **1.4.3 Cytokinin biosynthesis**

The level of biologically active cytokinin in an organ is regulated at diverse steps, including biosynthesis, activation, conjugation and degradation. Cytokinin biosynthesis has not been fully characterized, as biosynthesis and degradation of aromatic cytokinins remains to be elucidated (Sakakibara, 2006) but it probably differs from that of the isoprenoid type. In addition, the aromatic cytokinins have not been identified in many plants and it is unclear whether they are synthesised ubiquitously throughout the plant kingdom (Hirose et al., 2008). Isoprenoid cytokinins, on the other hand, exist in all plants so far studied (Tomaž and Marina, 2010).

Cytokinin biosynthesis is a multi-step process requiring the expression of several different enzymes (Mok et al., 1992). Two pathways for cytokinin biosynthesis have been suggested. One is the direct pathway, also called the *de novo* biosynthetic pathway. It involves formation of N<sup>6</sup>-isopentyladenosine monophosphate (iPMP), isopentyladenosine-5<sup>1</sup>-diphosphate (iPDP), or isopentyladenosine -5<sup>1</sup>-triphosphate (iPTP) from dimethylallyl pyrophosphate

(DMAPP) and AMP, ADP or ATP respectively. Another cytokinin biosynthesis pathway is the indirect pathway that involves binding of DMAPP to the adenine base of the anticodon region of tRNA. The key enzymes in both pathways are isopentenyl transferases, which can use either a free adenosine phosphate (AMP, ADP or ATP) or the adenosine moiety in a tRNA molecule (Kakimoto, 2001, Takei et al., 2001).

In the past few years, our understanding of cytokinin biosynthesis has greatly progressed due to the identification of key pathway genes encoding isopentenyl transferase (*IPT*), tRNA-isopentenyl transferase (tRNA-*IPT*) (Miyawaki et al., 2004), cytokinin trans-hydroxylase, CYP735A (Takei et al., 2004) and the cytokinin nucleoside 5-monophosphate phosphoribohydrolase, LONELY GUY (*LOG*) (Kurakawa et al., 2007).

In *Arabidopsis*, the initial step of iP and tZ biosynthesis is catalyzed by *IPT* using DMAPP and adenosine5-diphosphate (ADP), or adenosine 5-triphosphate (ATP), to generate iP-ribotides. These iP-ribotides are then hydroxylated to tZ-ribotides by CYP735A1 or CYP735A2 (Takei et al., 2004). On the other hand, biosynthesis of cZ is initiated by tRNA-*IPTs* that catalyze the prenylation of tRNA using DMAPP. However, the enzyme for hydroxylation has yet to be identified in plants. Conversion of iP, cZ and tZ-riboside 5'-monophosphates into their active forms occur by two pathways: the *LOG* and two-step pathways. In the former, cytokinin riboside 5'-monophosphates are directly converted to free-base cytokinins by *LOG* (Kurakawa et al., 2007). In the latter pathway, ribotides are dephosphorylated to the ribosides and subsequently converted to free-base cytokinins (Kristopeit, 1981).

Adenylate *IPTs* have been identified in higher plants (Kakimoto, 2001) and some phytopathogenic microorganisms such as *Agrobacterium tumefaciens* (Akiyoshi et al., 1984) and *Rhodococcus facians* (Crespi et al., 1992). In *A. thaliana*, seven *IPT* genes (*AtIPT1*, *AtIPT3*- *AtIPT8*) (Kakimoto, 2001, Takei et al., 2001) and in rice, eight *IPT* genes (*OsIPT1*-*OsIPT8*) (Tomoaki Sakamoto, 2006) were identified as being involved in the N-prenylation step of cytokinin biosynthesis using ADP/ATP as substrates.

The iP nucleotides produced by *IPT* in plants undergo hydroxylation at the prenyl side chain to synthesize tZ-nucleotides. In *Arabidopsis*, two cytochrome P450 monooxygenases, CYP735A1 and CYP735A2, catalyze this reaction (Takei et al., 2004). The CYP735As

utilize iP-nucleotides, but not the iP-nucleosides or iP. Since this reaction is stereospecific, the CYP735As do not produce cZ-nucleotides (Takei et al., 2004).

To become biologically active, cytokinin nucleotides produced by IPTs and CYP735As have to be converted to the free base form. Recently, a cytokinin activating enzyme named LOG that directly converts cytokinin nucleotides to the active nucleobases was identified in rice (Kurakawa et al., 2007). LOG has phosphoribohydrolase activity and releases a ribose-5' monophosphate moiety from cytokinin nucleoside 5' monophosphate. The name LOG comes from its mutant phenotype in which maintenance of the shoot meristem is defective and flowers often contain only one stamen but no pistil, thus LONELY GUY (Kurakawa et al., 2007). The phenotype showed the importance of the LOG-dependent cytokinin activation pathway in maintenance of the shoot apical meristem in rice.

Recent studies on the LOG family genes in rice and *Arabidopsis* suggest that production of cytokinin occurs in nearly all plant parts (Kuroha et al., 2009). Superimposition of the expression patterns for the *IPT* and *CYP735A* genes, in *Arabidopsis*, reveals the differential distribution of *de novo* synthesis pathways for iP and tZ. For instance, *AtIPT3* is expressed in phloem tissue in rosette leaves, whereas expression of *CYP735As* in rosette leaves is scarcely detectable (Kurakawa et al., 2007). Alternatively, both *IPTs* and *CYP735As* are expressed in roots. Such differential distribution of these cytokinin biosynthesis genes might be important to produce the various cytokinin species in underground and aboveground organs (Kuroha et al., 2009).

It has been recognized that the rate-limiting step in cytokinin biosynthesis is controlled by isopentenyl transferase (*IPT*) (Kakimoto, 2001) and that this enzyme is coded for by a gene family with members expressed specifically in different tissues and/or developmental stages (Miyawaki et al., 2004). Plants have two classes of isopentenyl transferases (*IPTs*) acting on the adenine moiety: ATP/ADP isopentenyl transferases (in *Arabidopsis thaliana*, *AtIPT1*, 3, 4–8) and tRNA *IPTs* (in *Arabidopsis*, *AtIPT2* and 9). ATP/ADP *IPTs* are likely to be responsible for the bulk of cytokinin synthesis, whereas it is thought that *cis*-zeatin (cZ)-type cytokinins are produced possibly by degradation of *cis*-hydroxy isopentenyl tRNAs, which are formed by tRNA *IPTs* (Emery et al., 1998).

Since there are multiple IPT genes in plant genomes, it is difficult to explain how their expression is regulated. In *Arabidopsis* (*Arabidopsis thaliana* L.), where all nine *AtIPT* genes have been characterised, a comprehensive study on their expression patterns has been made (Miyawaki et al., 2004). Results clearly showed that *Arabidopsis* IPT genes are highly tissue specific (Miyawaki et al., 2004) and that their expression is regulated by different developmental factors (Tomaž and Marina, 2010). Recently, the expression of *AtIPT4* and *AtIPT8* was quantified and localized in *Arabidopsis* seeds (Miyawaki et al., 2004, Belmonte et al., 2013) and *ZmIPT2* to the immature maize caryopsis (Brugière et al., 2008).

#### **1.4.4 Cytokinin degradation - cytokinin oxidase/dehydrogenase (CKX)**

The key enzyme of cytokinin degradation is cytokinin oxidase/ dehydrogenase, which irreversibly inactivates cytokinins by cleaving the bond between the purine ring and the side chain. The substrates of CKX are free cytokinin bases and ribosides with an unsaturated side chain (i.e. iP, iPR, *tZ* and *tZR*), Dihydro forms (DHZ-type) and *O*-glucosides cannot be degraded by CKX. In the case of *N*-glucosides, the data are somewhat contradictory and it is not yet unequivocally clear whether or not cytokinin *N*-glucosides are degraded by CKX (Bilyeu et al., 2001).

The products of iP or iPR degraded by CKX are 3-methyl-2-butenal and adenine, or adenine riboside respectively. Interestingly, the original name of the enzyme (cytokinin oxidase) might be inappropriate, because it seems that CKX is not a copper containing enzyme, but a flavoprotein that does not require oxygen for its activity (Werner et al., 2006). Therefore, dehydrogenase is a more appropriate description of this enzyme. There is evidence of tissue specific expression of different *CKX* genes, indicating that degradation of cytokinins, like their biosynthesis, is a well-regulated process (Werner et al., 2006).

It is believed that CKX is responsible for most metabolic cytokinin inactivation and plays an important role in maintaining cytokinin homeostasis in many plant species. Enzymes that encode CKX were firstly isolated from maize (Letham, 1994). Evidence shows that CKX genes belong to multigene family with at least seven homologues in *Arabidopsis*, eleven in rice, seven in barley, eight in wheat, and five in maize (Bilyeu et al., 2001). The different physiological functions remain under investigation.

It has been shown that manipulation of these genes provides a useful experimental approach for enhancing cytokinin catabolism in transgenic plants and studying the consequences of cytokinin deficiency.

#### **1.4.5 Cytokinins in developing seeds**

For many years, it was believed that cytokinin biosynthesis occurred only in roots (Letham, 1994). Subsequently, cytokinins were clearly demonstrated in chickpea and lupin xylem exudates (Emery et al., 2000, Wang et al., 2000), as well as in phloem exudates (Emery et al., 2000), indicating their translocation to other plant parts. In white lupin seeds and maize kernels more than 15 isoprenoid cytokinin metabolites could be detected (Mok et al., 2000).

High levels of cytokinins have been shown in developing seeds from the time Letham identified zeatin in *Zea mays* (Letham, 1963, Jameson et al., 1982). In developing lupin seeds cytokinin levels are extremely high compared with those in vegetative tissues (Davey and Vanstaden, 1979), and in cereal grains it is not uncommon for as much as a 500-fold transient increase in cytokinin to occur in the endosperm for just a few days following anthesis (Jameson et al., 1982, Brenner and Cheikh, 1995).

In developing seeds, concentrations of cytokinins exceeded those in other tissues by an order of magnitude (Emery et al., 2000, Rock and Quatrano, 1995). In addition, the seed cytokinins are subjected to significant temporal fluctuations. The endospermic fluid of white lupin seeds has the highest cytokinin concentrations ever reported for any plant species (Emery et al., 2000). Similarly, the highest cytokinin concentrations were detected in maize caryopsis between 6 and 10 days after pollination, especially in basal part of the caryopsis (Rijavec et al., 2011, Brugière et al., 2003). The concentration was 9000 and 16500 pmol/g tissue dry weight in the upper and basal part respectively (Rijavec et al., 2009). Seeds of some eudicots have large quantities of cis-type cytokinins (Emery et al., 1998, Quesnelle and Emery, 2007a). For example, in pea and lupin seeds, developmental processes are regulated by cytokinin cZ type phosphates and ribosides, which are also the predominant forms (Quesnelle and Emery, 2007b, Letham and Zhang, 1989).

The levels of endogenous cytokinin reported in rice vary among organs and developmental processes. The highest concentrations of cytokinins in rice are generally found in developing seeds (YANG et al., 2002). In a study conducted by YANG et al. (2002), zeatin (Z) and zeatin riboside (ZR) contents increased in the endosperms of spikelets during early endosperm development. Morris et al. (1993) reported that zeatin (Z) and zeatin riboside (ZR) in developing rice (*Oryza sativa*) and wheat (*Triticum aestivum*) grains showed large transient increases following pollination, which coincided with the period of seed setting and maximum endosperm cell division. This might be the reason why cytokinins are said to positively affect grain development. tZ type cytokinins are predominant type in rice seeds and hence they are believed to be involved in the regulation of early seed development (YANG et al., 2002).

The early evidence for the regulatory functions of cytokinins has been obtained by correlation between grain weight and the level of endogenous cytokinins in barley and wheat by Michael et al. (1970) and Herzog and Geisler (1982). The highest amounts of cytokinins were recorded at the early growth stages, viz., heading or anthesis or at milk stage, suggesting that the cytokinins may play important role in grain development (Takagi et al., 1989). Aside from its effect on rice grain-filling, cytokinins are known to be effective in retarding senescence which indirectly affects grain production.

In soybeans, cytokinins play a role in regulating flower and pod development (Reese et al., 1995). There is strong evidence supporting a role for cytokinins in the regulation of flowering and fruit set (Peterson et al., 1990, Reese et al., 1995). In particular, tZ and tZR abundance correlates with the rate of endosperm cell division, whereas iP and iPR abundance does not.

The origin of endogenous cytokinin during seed development was initially proposed to be from the roots (Nooden and Leopold, 1978). Then it was recognised that root-supplied cytokinin does not provide sufficient cytokinin for seed development (Nooden and Letham, 1984). However, recent studies on the spatial distribution of cytokinin biosynthesis genes has demonstrated that cytokinins are produced not only in roots, but also in various sites within the aerial parts of the plant. In *Arabidopsis*, the *IPT* genes are expressed in numerous organs including roots, leaves, stems, flowers, and siliques (Miyawaki et al., 2004).

#### **1.4.6. Cytokinin oxidase in seeds**

Metabolism of cytokinin is complex and involves irreversible deactivation by CKX as well as a complex array of glucose conjugations and it has also been shown to be both species- and tissue-specific (Jameson, 1994). CKX has a critical role in seed development which is clear from the work in which CKX is either over- or under-expressed in developing seeds (Kopečný et al., 2006). The importance of CKX was shown conclusively by Ashikari et al. (2005b). In their work, it was shown that reduced expression of *OsCKX2* causes cytokinin accumulation in inflorescence meristems and increase in the number of reproductive organs, resulting in enhanced grain yield.

It was also found that *ZmCKX1* is the most abundant cytokinin oxidase/dehydrogenase in developing maize kernels and other *ZmCKX* genes have been found to be expressed in developing kernels (Brugière et al., 2003). It was shown that at 8 DAP, *ZmCKX1* was expressed in the pedicel as well as the lower portion of the endosperm (Brugière et al., 2003). Various functions of cytokinin and in particular of *CKX3* and *CKX5* in regulating organ size of *A. thaliana* were revealed by Bartrina et al. (2011). It was found that size of the reproductive meristems of *Arabidopsis* was promoted by cytokinin (Bartrina et al., 2011).

### **1.5 Role of transporter genes**

In leaves, primary products of photosynthetic CO<sub>2</sub> fixation and nitrogen assimilation are loaded into the phloem and translocated, mainly as sugars and amino acids, to heterotrophic sink organs to support their growth and development (Lalonde et al., 1999). These mesophyll cells of mature leaves are net carbon exporters or source tissues. Whole plant productivity and crop yield depend on the distribution of photoassimilate between competing sink organs (Wardlaw, 1990). Source – sink relation and signaling play a crucial role in this stage.

Since starch and protein are the major forms of storage, sucrose transporters and amino acid transporters have an important role in nutrient transport. Genes and proteins catalyzing Suc/H<sup>+</sup> symport are called sucrose transporters (SUT) or sucrose carriers (SUC) (Riesmeier et al., 1994) and those participating in passive transport are called sucrose facilitators (SUF). Loading of sucrose into the phloem in source tissues is mediated by SUTs or SUCs (Stadler et al., 1995, Rennie and Turgeon, 2009). Unloading of phloem in the sink tissues seems to

occur both symplastically (Turgeon, 1996) and apoplastically (Wright and Oparka, 1989). In the second case, unloaded sucrose is hydrolyzed by cell wall-bound invertase and the product i.e., monosaccharides are transported into the sink tissues by specific hexose transporters (Chourey, 1992).

### 1.5.1 Sucrose transporters (SUT)

The analysis of sucrose transport in higher plants started more than 30 years ago (Giaquinta, 1980). It was not known then whether higher plants possessed only one gene for a sucrose transporter or two or more transporter genes were active simultaneously. Subsequently, many sucrose transporters have been cloned from different species (Sauer, 2007). The first SUT genes identified and sequenced were isolated from spinach (*SoSUT1*; *Spinacia oleracea*) and potato (*StSUT1*; *Solanum tuberosum*) (Riesmeier, 1993). Gradually, SUT genes were isolated from numerous species by sequence homology (Sauer et al., 1994). In *Arabidopsis*, all members of the SUT family are characterized as Suc/H<sup>+</sup> symporters. Subsequently, a class of SUT- Sucrose facilitators (SUF) were described and shown to express broadly in pea (*Pisum sativum*) and bean (*Phaseolus vulgaris*) (Zhou et al., 2007).

The long distance transport of sucrose occurs in the sieve elements of the phloem and is necessary for the carbon supply to photosynthetically inactive or non-green parts of plants such as roots, very young leaves, flowers, fruits and seeds. Evidence has shown that loading of sucrose into phloem is catalyzed by active, energy –dependent transporters. These transporters are located in the plasmalemma of the companion cells or sieve elements (Patrick and Offler, 2001).

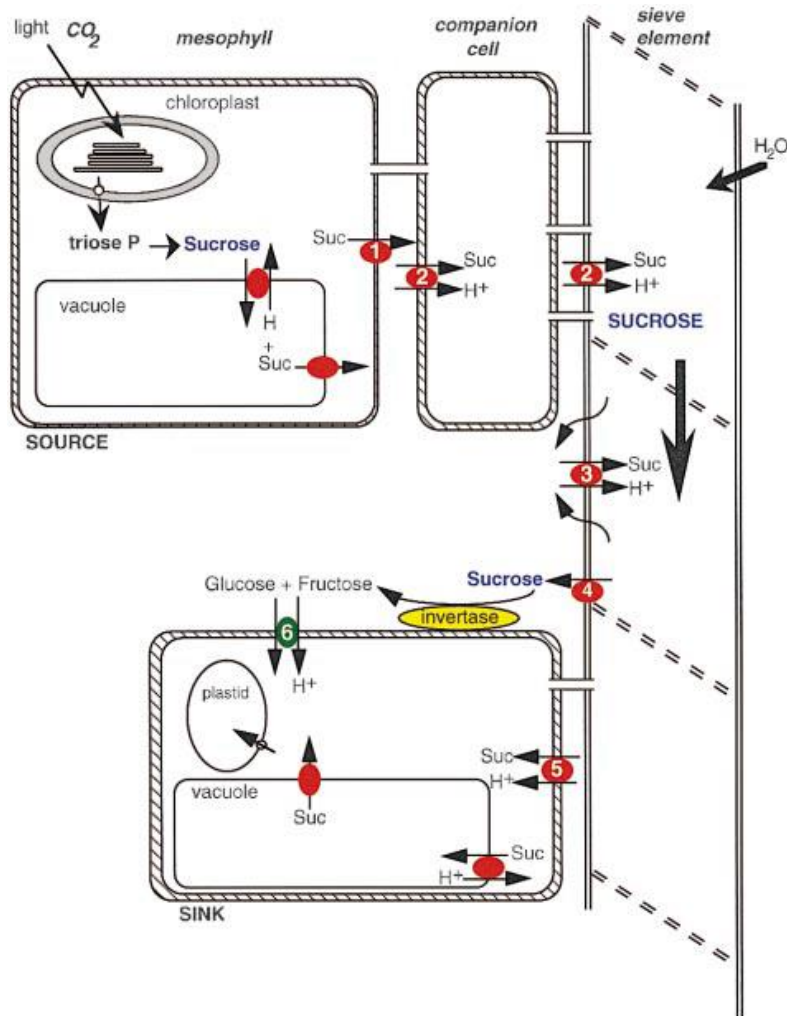
For developing seeds of grain legumes, photoassimilates released to the seed apoplasm from maternal seed coats are retrieved by abaxial epidermal and sub-epidermal cells (dermal cell complexes) of cotyledons followed by symplasmic passage to their underlying storage parenchyma cells (Weber et al., 1997) (Fig 1.4). In some species, the cells of these complexes differentiate into transfer cells (e.g. in broad bean and pea) (Patrick and Offler, 2001).

Transfer cells are ubiquitous in plants and important for nutrient transport. They are characterized by secondary wall ingrowths, resulting in an increased plasma membrane surface enriched in assimilate transporters (Offler et al., 2003). Transfer cell formation in seeds is an early differentiation event (Thompson et al., 2001).



Sucrose is a major component of the photoassimilates delivered to the cotyledons (Patrick and Offler, 2001) and its uptake depends on external sucrose concentrations (Patrick and Offler, 2001, Weber et al., 1997). Since sucrose is a large, polar solute, it requires proteins to facilitate efficient movement across membranes. Physiological studies support active transport energized by the proton motive force and facilitated diffusion across both the tonoplast and the plasma membrane. For energized transport, Suc/H<sup>+</sup> antiport to move sucrose into vacuoles is supported by physiological studies and Suc/H<sup>+</sup> symport to move sucrose out of vacuoles is supported by molecular evidence (Riesmeier et al., 1994). Thereby, sucrose reaches concentrations of several hundred millimolar up to more than one molar in the conducting vascular cells while extracellular sucrose concentrations range between 2 and 7 millimolar (Lohaus and Fischer, 2002). Sucrose/H<sup>+</sup> symporter transcripts and proteins have also been localized in sink tissues (Riesmeier et al., 1994, Kühn et al., 1997). Hence, it is suggested that sucrose transporters are also involved in sucrose release from the phloem into the apoplast (Christina Kühn 2003, Roberto Viola, 2001) and/or that they contribute to the uptake of sucrose into sink tissues such as pollen, seeds, and the vascular tissues (Weber et al., 1997, Hirose et al., 2010). The hydrostatic pressure difference between source and sink tissues established by sucrose transporters drives the mass flow of water and nutrients in the phloem vessels (Rae et al., 2005).

Members of the SUT family cluster into distinct clades and these are useful in forming hypotheses on function. Aoki and colleagues identified three distinct branches (Type I, II, and III) in one of the earliest comprehensive trees (Aoki et al., 2003). Similarly, Lalonde and colleagues used clades I, II, and III (Lalonde et al., 2004). These trees have the same basic structure, but the type/Clade naming is not consistent. As more sequences were identified, the number of major branches increased from three to four (Sauer, 2007), to five (Slewinski, 2009, Kühn and Grof, 2010), as the type II (Clade III) branch underwent further sub-division. However, inconsistency in nomenclature remains.



**Figure 1.4 Long-Distance Sugar Transport by the Phloem.**

Copied from *The Plant Cell*, Vol. 11, 707–726 (Lalonde et al., 1999). From mesophyll, sucrose may be loaded into the SE/CC complex either through plasmodesmata or via the apoplast. The apoplasmic loading mechanism requires sucrose export (1) from the mesophyll or the vascular parenchyma and reuptake (2) into the SE/CC complex. Hydrostatic pressure drives phloem sap movement toward sink tissue. Passive leakage can take place along the path (indicated by wavy arrows). Reuptake (3) also occurs along the path of the phloem. Apoplasmic phloem or post phloem unloading necessitates a sucrose exporter at the sink tissue (4). Import of sucrose and other solutes into sink tissue may occur through plasmodesmata or sucrose transporters (5). In addition to plasmodesmal and transporter-mediated uptake, cells in the sink may take up sucrose, subsequent to its hydrolysis by an apoplasmic invertase, as hexoses (6). The vacuolar transport system could consist of a H<sup>+</sup>/sucrose antiporter for uptake and a uniporter for release.

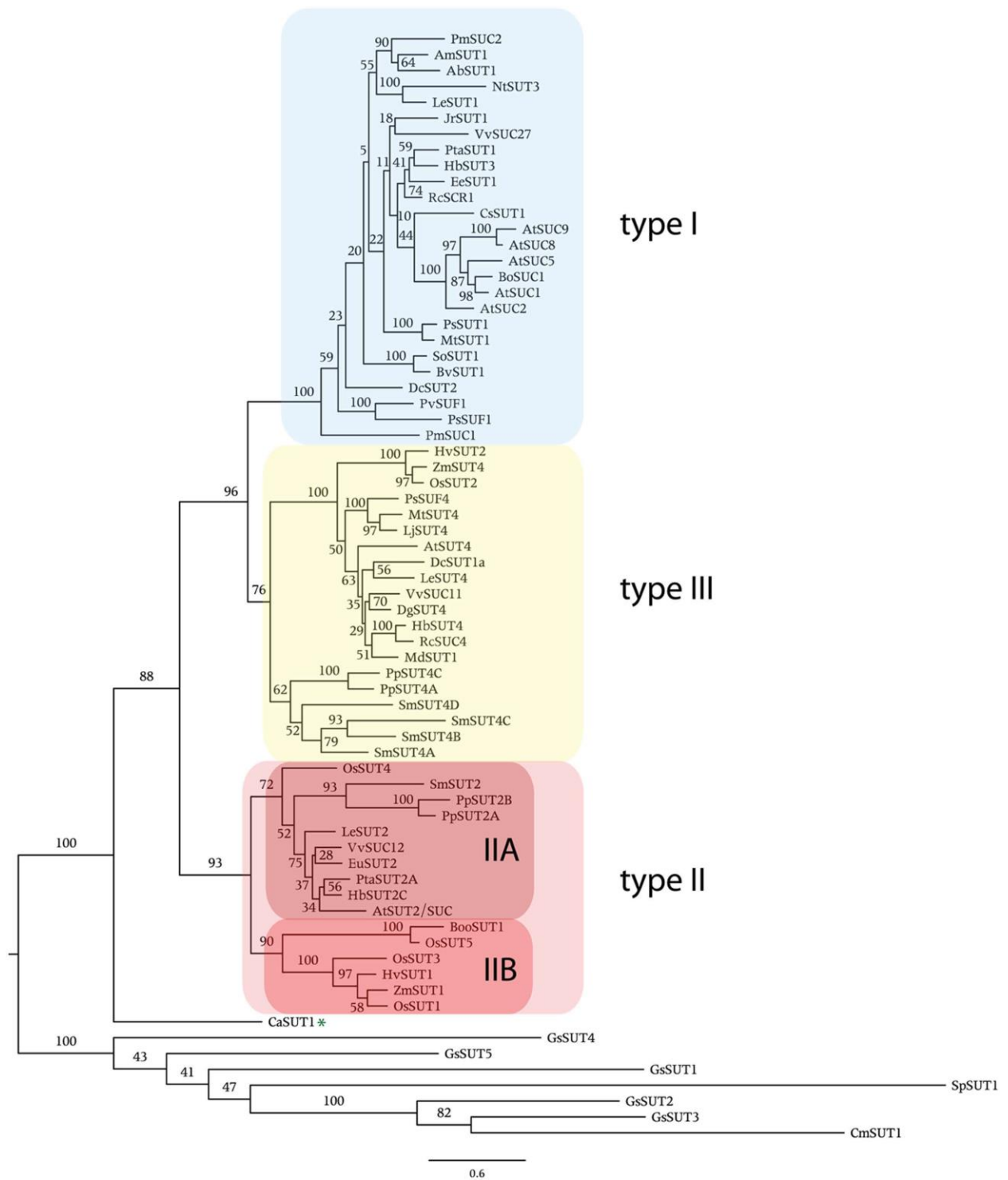
The latest phylogenetic classification was done by Reinders et al. (2012), in which they followed the nomenclature of Aoki et al. (2003). It showed the presence of three main groups

of SUTs: Type I, II and III (Fig 1.5). Interestingly, Type I SUTs are found only in eudicots. Type I SUTs are necessary for essential functions in eudicots such as phloem loading (Riesmeier et al., 1994, Gottwald et al., 2000) and normal pollen function (Sivitz et al., 2008). Type II and III SUTs are present in all land species. Type II has an important role in phloem loading in monocots (Slewinski, 2009).

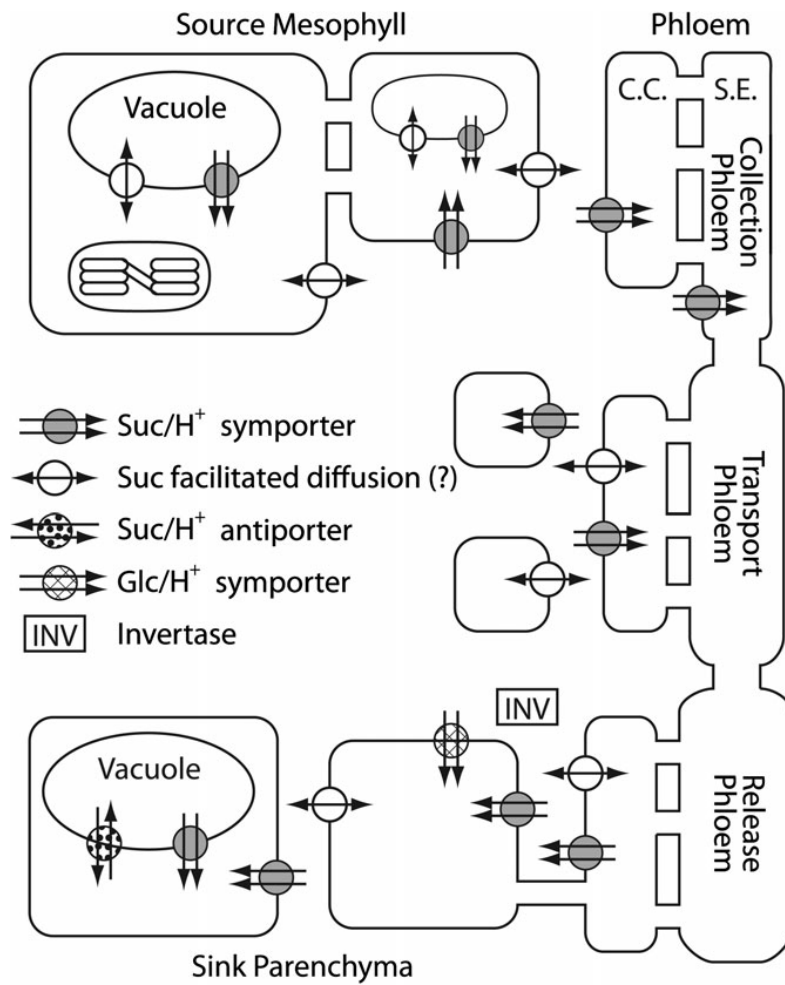
All Type I SUTs appear to localize to the plasma membrane and have moderate affinity for sucrose. Type I SUTs are thought to have the highest specificity for sucrose among the SUT groups (Sun et al., 2010). In *A. thaliana*, Type I SUTs show specificity in both expression and transport function. *AtSUC2* is necessary for loading sucrose into the phloem (Gottwald et al., 2000).

Type II SUT sequences were identified in eudicots, monocots, non-vascular land plants and vascular plants. All Type II SUTs are localized in plasma membrane. The Type II SUTs were further divided into two subgroups Type IIA and type IIB. These two were previously identified by Slewinski (2009). There is a structural difference between Type IIA and IIB SUTs. Type IIA proteins have a longer central cytoplasmic loop compared to Type IIB SUTs. Each angiosperm genome appears to have one gene in the Type IIA subgroup. Type IIB subgroup is monocot specific. Rice encodes three Type IIB transporters (*OsSUT1*, *OsSUT3* and *OsSUT5*). Members of this group are monocot phloem loaders. *ZmSUT1* has been shown to be expressed in vascular tissue and to function in phloem loading (Slewinski, 2009).

The first Type III SUTs were isolated from *Arabidopsis*, tomato, potato and barley and named as *AtSUT4*, *LeSUT4*, *StSUT4* and *HvSUT2* respectively (Weise et al., 2000). Type III SUTs are roughly 47% similar to Type I SUTs and have approximately 10-fold lower affinity for sucrose (Reinders et al., 2008). Because of this, they were described as low affinity/high-capacity transporters. Members of Type III SUTs localise to the tonoplast. Each angiosperm genome appears to contain a single type III SUT gene. Type III SUTs will transport a greater variety of substrates than type II SUTs, but Type II are less specific than in Type I (Reinders et al., 2008).



**Figure 1.5 Phylogenetic analysis of plant sucrose transporters and homologs.**  
Copied from *frontiers in Plant Science*, (Reinders et al., 2012)



**Figure 1.6 Diagram of sucrose membrane transport in different parts of the plant.**  
 Copied from Molecular Plant • Volume 4 • Number 3 • Pages 377–394 (Brian, 2011)

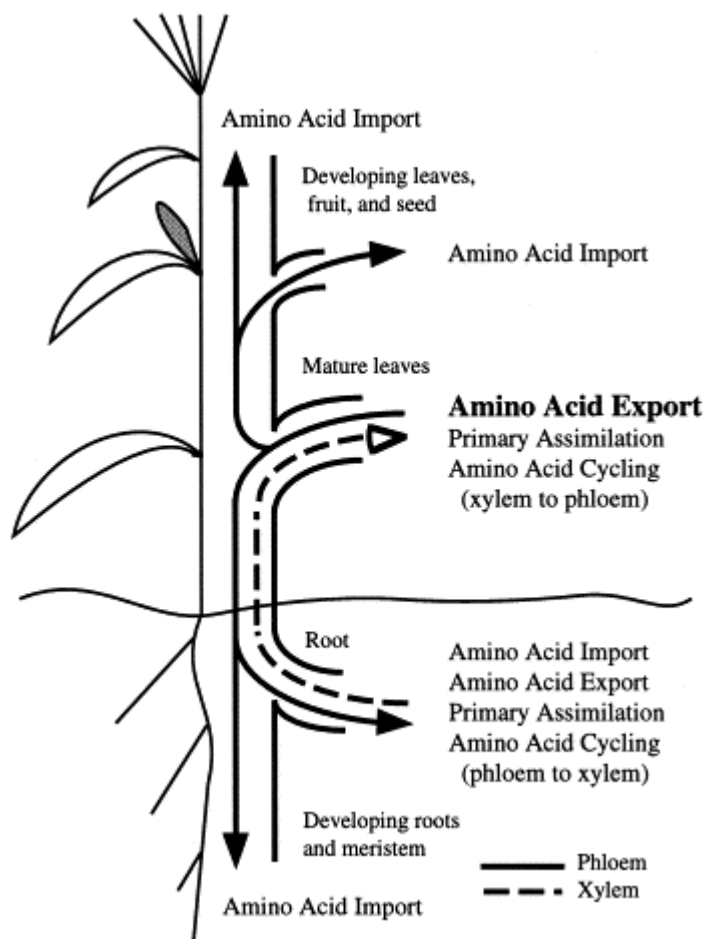
In the cytoplasm, sucrose whether synthesized or imported, can be compartmentalized to vacuoles and other organelles (Fig 1.6). Vacuoles store excess sucrose during the day and export it at night (Martinoia et al., 2000; Neuhaus, 2007).

### **1.5.2 Amino Acid Permease transporters (AAP)**

Physiological and genetic evidence demonstrates that transport systems are responsible for the uptake and transfer of amino acids within plants (Fischer et al., 1998). Amino acid transporters have been identified in various plant species and it is clear that they exist as members of gene families with transport properties (Rentsch et al., 2007). Synthesis of seed proteins relies on importation of organic nitrogen mainly as amino acids, into the developing embryo which is comprised of two large cotyledons (Rainbird et al., 1984). The cotyledons are strong sinks for amino acids for growth and storage protein synthesis and they import amino acids from surrounding maternal seed coat tissue (Thorne, 1985) via the apoplast. Amino acid absorption has been studied extensively in leguminous seeds, such as pea (*Pisum sativum*) and soybean (*Glycine max*) (Lanfermeijer et al., 1990, de Jong and Borstlap, 2000).

Amino acids are delivered to developing grain legume seeds almost exclusively in the phloem (Fig 1.7), assuming that transfer occurs along the path from xylem to phloem (VAN BEL, 1990). Unloading of phloem in the seed coats is considered to be symplasmic (OFFLER et al., 1989, OFFLER and PATRICK, 1993). De Jong and Wolswinkel (1995) found that releases of amino acids from seed coats occurs by a facilitated membrane transport mechanism, probably through non-selective pores (De Jong, 1997). The released nutrients are taken up by the developing embryos from the seed apoplast. In pea cotyledons, a passive transport was demonstrated by Lanfermeijer et al. (1990). The saturable uptake component for sucrose and amino acids seems to be proton motive force coupled (Tegeder et al., 1999). In developing fava bean (*Vicia faba*) and pea seeds, the inner surface of the seed coats and external epidermis of the enclosed cotyledons are made of transfer cells (OFFLER and PATRICK, 1993, Tegeder et al., 1999). These transfer cells are probably the principal sites for the exchange of sucrose amino acids across the extracellular space between seed coats and cotyledons (Harrington et al., 1997a, Harrington et al., 1997b, Weber et al., 1997, Tegeder et al., 1999).

A number of amino acid transporter genes have been identified in *Arabidopsis* (Fischer et al., 1998). Based on sequence homology, these plant transporters are classified into two major families: the ATF (Amino acid Transporter Family) and APC family (amino-acid-polyamine-choline facilitator) (Fischer et al., 1998). The ATF is also referred to as the Amino Acid Permease (AAP) family. The ATF family consists of the broad-specificity AAP transporters (Amino acid permease), the basic amino acid LHT (lysine-histidine-like) transporters, the compatible solute ProT (proline) transporters and the putative AUX (auxin) transporters (Tegeder and Ward, 2012).

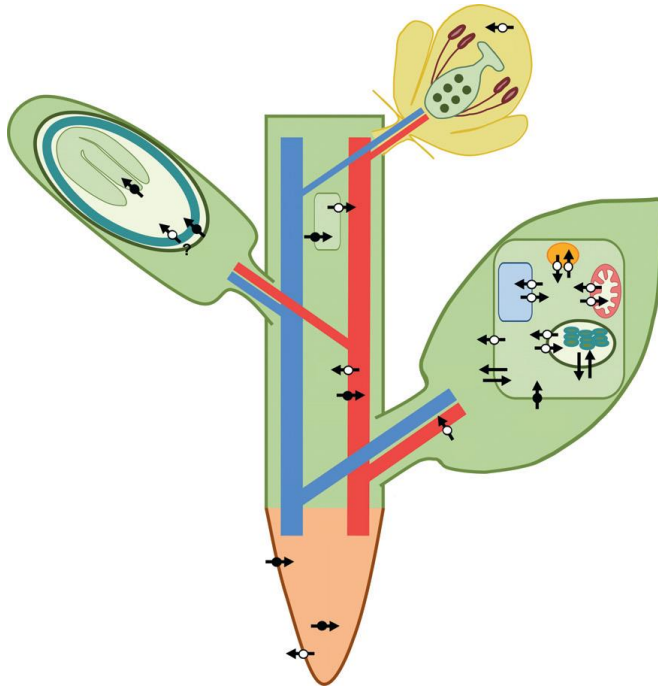


**Figure 1.7: Schematic representation of amino acid transport between the sites of primary nitrogen assimilation and the import dependent tissues.** Copied from Amino acid transporters in plants, (Biochimica et Biophysica Acta (2000) pp 275-280).

The AAPs are encoded by multigene families which display temporal and spatial expression patterns (Okumoto et al., 2002). Eight *Arabidopsis* AAPs were isolated. Of these *AtAAP8* and *AtAAP6* have been identified as high affinity amino acid transport systems. *AtAAP6* functions in uptake of amino acids from xylem. *AtAAP6* is expressed in xylem parenchyma and *AtAAP8* is expressed in siliques and developing seeds, indicating its role in supplying organic nitrogen to developing seeds (Okumoto et al., 2002).

ATF was first described in plants and comprises 46 members in *Arabidopsis* (Schwacke, 2003). Translocation of amino acids in support of developing seeds must be integrated between source and sink, and transporters are involved at both ends of the transport pathway. In developing seeds, amino acid transporters could be involved at different levels, but they most likely function in uptake by cotyledons from the apoplastic space (Tegeder et al., 2007). Two pea amino acid transporters, *PsAAP1* and *PsAAP2* were identified by Tegeder et al. (2000) and their result demonstrated *PsAAP1* expression in developing pea seeds, specifically in seed coats and cotyledons, whereas, *PsAAP2* expression could not be detected. A specific function of *AtAAP1* in loading amino acids into the embryo is shown in Fig 1.8. However, when the *AtAAP1* promoter linked to *PsAAP1* is expressed in pea, expression was less specific and observed both in the phloem and the seed cotyledons (Tegeder et al., 2007). Tegeder (2012) suggested that AAP expression generally was not as specific as first thought. This was confirmed by Song et al. (2015).





**Figure 1.8: Overview of amino acid transporters with demonstrated functions in plants** (arrow with black circle and two black arrows). This includes transporters involved in (i) amino acid uptake into root cells [LHT1, AAP1, AAP5, ProT2 and CAT6], (ii) import into mesophyll cells [LHT1], the endosperm [AAP8], embryo [AAP1], xylem parenchyma [AAP6] and transport phloem [AAP2], (iii) cellular efflux of glutamine and histidine, and uptake of aspartate and glutamate [SiAR1] and in (iv) glutamate/ malate exchange across chloroplast membranes [DiT2.1]. However, many amino acid transport systems still need to be discovered and their physiological function elucidated (arrow with white circle). Examples for these are transporters functioning in phloem loading of leaf minor veins for N source–sink partitioning, or in import of amino acids into floral cells/tissue in support of reproduction. In addition, cellular export systems for specific amino acids or with broad substrate specificity as well as intracellular transporters (e.g. vacuole, chloroplast, peroxisome and mitochondria) remain to be identified and analyzed with respect to their physiological significance. Copied from Current opinion in Plant Biology (Tegeder, 2012)

## 1.6 Role of cell wall invertase genes

Sucrose is the major form of photoassimilates transported from photosynthetically active tissues such as mature leaves to non-photosynthetic tissues such as flower, fruit, seed and root. Once it has reached these sink tissues, sucrose must be degraded into hexoses or their derivatives for numerous biosynthetic and metabolic processes (Sturm, 1999). In higher plants, there are two enzymes which catalyze the degradation of sucrose, they are sucrose synthase (SuS) and invertase (INV). Sucrose synthase converts sucrose into UDP-glucose and fructose in the presence of UDP, whereas invertase cleaves sucrose into its monosaccharides, glucose and fructose (Chourey, 1992, Roitsch and González, 2004).

Invertases are classified based on their optimum pH, solubility and sub-cellular locations. The three different groups are vacuolar (VIN), cell wall or apoplastic (cwINV) and cytoplasmic (CIN) (Sturm, 1999). Vacuolar and cell wall invertase have some common biochemical properties, even though they are located in different compartments. Both cleave sucrose most effectively at pH around 4.5- 5.5 (Sturm, 1999), whereas cytoplasmic invertase has an optimum pH of 7.0- 7.8 and hydrolyzes sucrose in the cytosol. The first cloned plant invertase was cell wall invertase from carrot (Sturm and Chrispeels, 1990). Later, the gene for cell wall invertase (cwINV) has been isolated from a variety of species including tomato, *Arabidopsis* and maize.

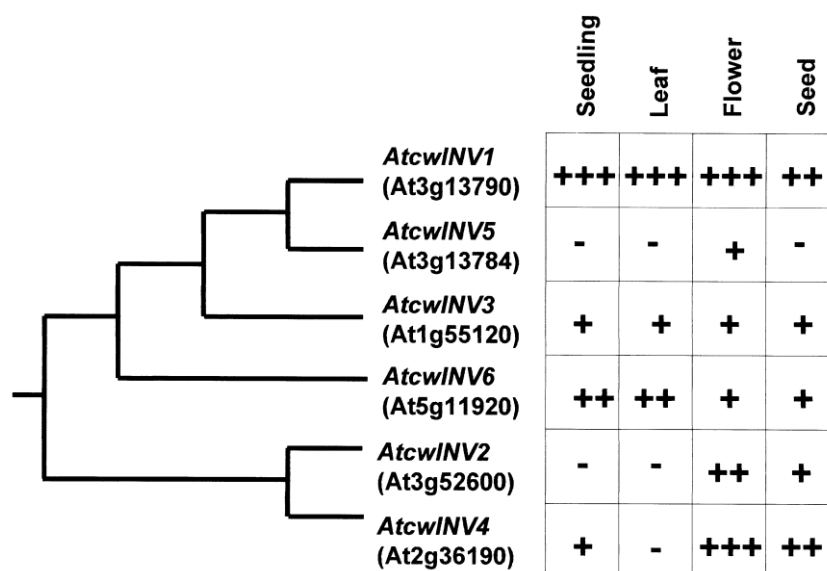
Cell wall invertases (cwINV) play a crucial role in metabolic activities and development of reproductive organs. They may also have important roles in phloem loading (Roitsch and González, 2004) and cell division (Weber et al., 1996). Cleavage of sucrose into glucose and fructose could greatly increase the osmotic pressure of cells, suggesting a possible function of cell wall invertase in cell elongation and plant growth (Gibeaut et al., 1990). Recent evidence shows that cwINV is involved in the earliest phases of flower development. The activity of cwINV supplies hexoses to the developing anthers and ovaries before pollination occurs (Zhang et al., 2006). At the same time, co-expression of cwINV and hexose transporters has been observed in many systems including developing fruits (Hayes et al., 2007) and seeds (Weber, 2005).

Evidences show that silencing the expression of a cwINV, Lin5, in tomato reduced pollen

viability and elongation, and consequently seed number (Zanor et al., 2009). Also, it was reported that elevation of endogenous cwINV activity significantly increased grain size in rice (Wang et al., 2008). These findings show that the development of seed and fruit is also regulated by cwINV activity in these crops.

High activities of VIN and cwINV are seen in the maternal tissue or seed coat of young seeds of legumes (Weber et al., 1995). In general, these two are expressed sequentially (Andersen et al., 2002). cwINV facilitates nutrient unloading and thereby increases the concentration gradient of sucrose. cwINV are very active in growing zones and expanding sinks (Weber et al., 1995). In *V. faba*, *VfcwINV* was highly expressed in the unloading zones, cleaving the incoming sucrose and hence creating a high hexose environment during the cell division phase (Weber et al., 1995).

In *A. thaliana*, six putative *cwINV* genes have been identified. Out of these, five genes are expressed in the developing seeds, and of these, four seems to be expressed more strongly in the cell division phase (Fig 1.8) (Sherson et al., 2003, Song et al., 2015).

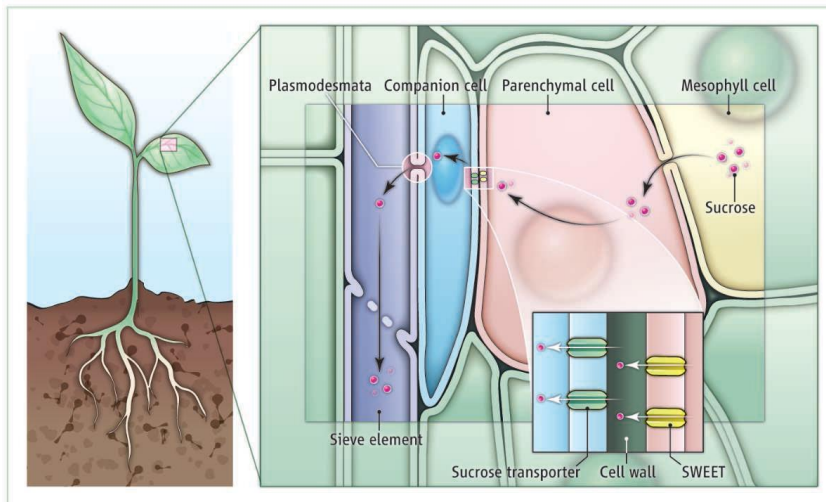


**Figure 1.9: The Arabidopsis cwINV gene family.** (+) expression, (-) no expression detected. Copied from Journal of Experimental Botany, Vol. 54, No. 382, pp. 525-531, January 2003.

Studies have shown different important roles of *cwINV* in seed filling, probably through regulating sucrose unloading and the establishment and maintenance of sink strength (Weber et al., 1996, Wang et al., 2008, Jin et al., 2009, Zanol et al., 2009). However, it remains largely unknown how *cwINV* exerts its regulation early in seed development (Ruan et al., 2010).

## **1.7 Role of SWEET (Sugars Will Eventually be Exported Transporter) genes in seed development**

Sugars, the primary products of photosynthesis, are synthesized in mesophyll cells and need to be transported to heterotrophic tissues for the growth and development of the plant (Lalonde et al., 2004). It is believed that phloem loading is the initial step in the transport pathway. Two major phloem loading mechanisms have been proposed: apoplastic loading and symplasmic loading (Turgeon and Medville, 2004). In the case of apoplastic loading, sucrose transporters (SUTs) have been identified as the essential factors for sucrose translocation (Lalonde et al., 2004). Recently, a new class of sugar transporters, the SWEETs have been identified as a prerequisite for SUT- mediated phloem loading (Chen et al., 2012a) that facilitate diffusion of sugars across cell membranes down a concentration gradient. By using optical sucrose sensors, Chen et al. (2012b) identified a subfamily of SWEET, *AtSWEET11* and *12* that localised to the plasma membrane of the phloem (Fig 1.10).



**Figure 1.10: Sucrose partitioning in plants.** Sucrose is synthesized in leaf mesophyll cells and diffuses through plasmodesmata into phloem parenchyma cells. SWEET proteins facilitate sucrose efflux into the cell wall (apoplast). Downloaded from [www.sciencemag.org](http://www.sciencemag.org), on June 16, 2012.

It was revealed from phylogenetic analysis that SWEETs are prevalent in plants. There are 17 SWEET members in *Arabidopsis* and 21 in rice (Chen et al., 2012a, Chen et al., 2010, Yuan and Wang, 2013) falling into four clades. More recently, it was found that members of clade III preferentially transport sucrose across the plasma membrane (Chen et al., 2012b, Brian, 2011). Some SWEETs were characterised as bidirectional, pH independent and low-affinity sucrose transporters.

Evidence showed that *AtSWEET11* and *12* shared high sequence similarity and both were expressed in phloem parenchyma cells, which are adjacent to the SE/CC complex where phloem loading occurs. Thus, their involvement in the process of apoplastic phloem loading was strongly indicated (Chen et al., 2012b). The key role of these gene family members in phloem loading was supported by another study in mutant plants (Chen et al., 2012b). These findings shed light on a significant breakthrough in the concept of phloem transport and the regulatory mechanism of sucrose transport from source leaves to sink tissues.

## 1.8 Interaction between cytokinins and transporter genes

Legume seeds are a major source of plant-derived proteins. Storage protein accumulation in legumes occurs in the embryo during maturation where nutrients like sugars and nitrogen confer regulatory control on storage activities (Weber, 2005). Also, storage protein accumulation in pea depends on nitrogen availability in the seed (Miranda et al., 2001). It has been shown that the level of sink demand for carbon and nitrogen can feed back through uptake, translocation, and assimilation rates (Peoples and Gifford, 1990). To improve grain production in terms of yield, plants have to take up and accumulate more N and should also be able to use this N for grain growth and storage (Sinclair, 1998). In legumes, seed protein accumulation can be regulated by the capacity of the seed to import amino acids via specific transporters. In *V. faba* seeds, sink strength for nitrogen is acquired during maturation and is associated with amino acid transport. Evidence showed that overexpressing *VfAAP1* in *V. narbonensis* seeds increased seed strength for nitrogen and led to higher seed protein concentration (Rolletschek et al., 2005b). And then later, it was shown in another study that in transgenic AAP-12 seeds, cytokinin levels were dramatically higher (Gotz et al., 2007) which indicates the involvement of cytokinins in the N-mediated growth stimulation. Similarly, the analysis of pea embryos also reveals that there is a strong growth-promoting effect for cytokinins (Quesnelle and Emery, 2007a). Cytokinins have been shown to stimulate unloading of photo-assimilates from excised bean seed coats (Clifford et al., 1986). Moreover, in another study, it was also shown that cell wall invertase transcript levels were highly up-regulated by physiological concentrations of cytokinins (Roitsch and Ehneß, 2000), and the similarity in expression timing and localization of cwINV (Chourey, 1992) and *ZmIPT2* in maize kernels was interesting.

## 1.9 Aims and Objectives

Until recently plants ectopically expressing either an AAP or a SUT gene had not shown increased yield (Rosche et al., 2002, Rolletschek et al., 2005b). Under the auspices of the Advanced Seed Production Systems programme, Plant& Food Research had been developing transgenic peas overexpressing both an AAP and a SUT gene with the aim of enhancing yield and yield components, by stimulating transport of both C and N into the seed. Concurrently, and within the same programme, research on the cytokinins was being carried out on other

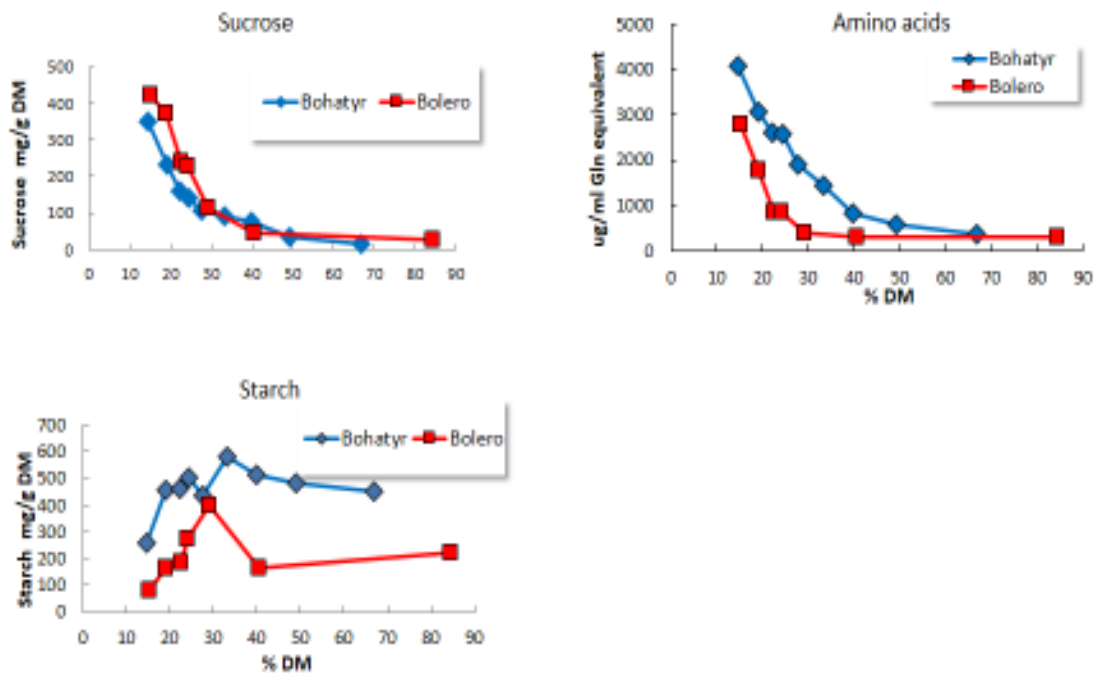
crops to identify gene family members likely to be influencing seed development (O'Keefe et al., 2011a, Song et al., 2012, Song et al., 2015).

In this thesis, two different cultivars of peas (*Pisum sativum*) were compared: Bolero and Bohatyr. Bolero is a process pea which is harvested immature and used for frozen peas and baby peas. The NZ industry mainly focuses on process peas. The plants exhibit semi-determinate growth, i.e. they are dwarfed and bushy. The seeds of Bolero are green and wrinkled when mature. Bolero seeds are harvested between 20-23 DAF, when the dry matter is less than 25%, and before the storage phase starts (Fig 1.11). Consequently, the harvest window is very narrow, being only 2-3 days under Canterbury conditions. Growers need a wider harvest window and the industry desires sweeter peas. The number of seeds is important but not the size. In contrast, Bohatyr, the field pea, is harvested at maturity as the seeds are mainly used for stock feed and for humans as mushy peas. The plant is long-strawed and normal-leaved with medium stem strength. The seeds are round and yellow when mature, and have a higher starch content than process pea at maturity (Fig 1.11). The industry is interested in increased protein and more, large seeds (Jan E Grant, personal communication).

The transgenic plants were previously developed lines of process and field pea, over-expressing a pea sucrose transporter (*PsSUT1*) and/or amino acid permease (*PsAAP1*) under the control of either the 35S promoter or a tissue specific promoter (*AtAAP1*). Following the initial single transformations and crossing, four generations of back cross populations were developed by Plant & Food Research. To analyse the phenotypic characters of parental, single and double transgenics, the plants were grown in the Lincoln University Biotron on two different occasions. On the first occasion, developing pods and leaves were collected and provided the material used in this thesis.

The phenotypic analysis was done by Dr Jan Grant at Plant & Food Research Institute, Lincoln. The results showed that there was a significant increase in the total number of seeds in double transgenic lines when compared with their parental wild type. Out of six double homozygotes, two lines have shown a consistent increase in seed number (Table 1.1). No change in the sucrose or amino acid levels were shown when compared to the parental lines, although the process pea showed greater starch in seeds at harvest (Jan E Grant, personnel

communication). As seed number was increased and seed size maintained or increased, the focus of this thesis was to assess the effect of the transgenes on the expression of genes involved in source/sink relationships.



**Figure 1.11: Sucrose, starch and amino acid content in the developing cotyledons.** A and B is sucrose and starch in mg/g of dry matter against % dry matter. C is the amino acid content in μg/ml of glutamine equivalent against % dry matter. Bohatyr = Field pea, Bolero = Process pea. (The analyses were completed by Plant& Food personnel. Data courtesy of Jan Grant).



Table 1. 2 Phenotypic analysis of yield components. The data are from two independent experiments. The analyses were completed by Plant& Food personnel. (Data courtesy of Jan Grant).

		100 seed weight g average	ave seed no average	% increase cf wildtype average	% increase cf recurrent parent average	seed per pod average	average no of pods/plant average
Line	<b>recurrent/donor</b>						
<b>Bolero</b>	<i>(process pea)</i>	<b>23.59</b>	<b>67.67</b>			<b>3.9</b>	<b>18.00</b>
<b>104</b>	<i>(sucrose transporter- process pea)</i>	<b>22.19</b>	<b>78.92</b>	<b>17%</b>		<b>4.3</b>	<b>20.00</b>
<b>1/55</b>	<i>(constitutive amino acid transporter)</i>	<b>28.20</b>	<b>66.88</b>	<b>12%</b>		<b>3.0</b>	<b>25.38</b>
<b>382</b>	<b>104/1/55</b>	<b>23.70</b>	<b>71.67</b>	<b>6%</b>	<b>-9%</b>	<b>5.0</b>	<b>16.92</b>
<b>242</b>	<b>104/1/55</b>	<b>20.68</b>	<b>78.58</b>	<b>16%</b>	<b>0%</b>	<b>4.8</b>	<b>19.00</b>
<b>562</b>	<b>104/1/55</b>	<b>23.34</b>	<b>89.83</b>	<b>33%</b>	<b>14%</b>	<b>4.3</b>	<b>21.33</b>
<b>8298</b>	<b>104/2/58</b>	<b>23.57</b>	<b>71.67</b>	<b>6%</b>	<b>-9%</b>	<b>3.7</b>	<b>20.92</b>
<b>Bohatyr</b>	<i>(field pea)</i>	<b>28.74</b>	<b>59.88</b>			<b>2.6</b>	<b>27.13</b>
<b>2/58</b>	<i>(seed specfic amino acid transporter)</i>	<b>28.05</b>	<b>59.13</b>	<b>-1%</b>		<b>3.3</b>	<b>24.00</b>
<i>field pea types</i>							
<b>5156</b>	<b>2/58/104</b>	<b>31.09</b>	<b>143</b>	<b>139%</b>	<b>142%</b>	<b>3.6</b>	<b>39.88</b>
<b>4241</b>	<b>2/58/104</b>	<b>30.73</b>	<b>81</b>	<b>35%</b>	<b>37%</b>	<b>3.3</b>	<b>25.50</b>

Although recent evidence clearly shows that cytokinin levels and activity of isopentyl transferase (*IPT*) and cytokinin oxidase (*CKX*) are tightly regulated, little work has been done to characterise this regulation pea. One aim of this thesis was to study the interaction between the transporter genes and cytokinin regulatory genes during different stages of pea seed development, by identifying key gene family members which may play an important

role in seed development in pea, and analysing the expression of these genes. More specifically, the focus was on describing the expression patterns of cytokinin biosynthesis and degradation genes (*IPT* and *CKX*), and nutrient transporter (*SUT* and *AAP*), invertase (*INV*) and SWEET (*SW*) genes across different developmental stages and tissue types to assess their temporal and spatial expression patterns in the two pea cultivars, representing a process pea (Bolero) and a field pea (Bohatyr). This was done to test the hypothesis that different gene family members differ in their spatiotemporal expression and that specific members of a gene family may play crucial roles in seed development and yield increase of peas. Gene expression of each family member was also studied in younger and older source leaves to analyse the source- sink relationships during the development of seed. The effect of the transgenes on gene expression of the WT and parental lines was then analysed. The objectives of this research were:

1. To identify the double homozygous seed lines of transgenic BC4 family, developed by Plant & Food Research Institute.
2. To identify and isolate members of cytokinin homeostasis genes (*IPT* and *CKX*) and transporter genes in pea (*Pisum sativum*) by BLAST searching available sequences of *A. thaliana* and all the members of these multi-gene families.
3. To isolate members of invertase (*PsINV*) and SWEET (*PsSW*) gene families in pea. To determine *in planta* expression levels of *PsIPT*, *PsCKX*, *PsAAP*, *PsSUT* and *PscwINV* gene family members during different stages of seed development in parental and transgenic lines with altered transporter genes (*PsSUT1* and/or *PsAAP1*) to understand their roles in seed development.
4. To determine *in planta* expression levels of *PsIPT*, *PsCKX*, *PsAAP*, *PsSUT*, *PscwINV* and *PsSW* gene family members in younger and older source leaves of WT, parental and transgenic lines to understand the roles of these genes in sink/source relationships.
5. To examine starch and sugar levels in the early developmental stages of pods and seeds, and in the seed coats of seed from both transgenic and non-transgenic pea plants in order to determine the effect of the transgenes on source/sinks relationships.

The overall aim of the thesis was to gain an understanding of the changes in gene expression that led to increased seed number and yield of those transgenic plants expressing both an *AAP* and a *SUT* transporter gene.

## CHAPTER 2

### Materials and Methods

One of the principal aims of this project was to study the effect of transporter genes during different stages of seed development. Two different cultivars of wild type peas, a field pea cultivar Bohatyr and a process pea cultivar Bolero, and transgenic peas were studied.

Transgenic plants were previously developed lines of process and field pea, over-expressing a pea sucrose transporter gene (*PsSUT1*) and/or amino acid permease gene (*PsAAP1*) under the control of either the 35S promoter or a tissue specific promoter of *Arabidopsis thaliana* amino acid permease gene *AtAAP1*. Following the initial crossing of the two single transformants, four generations of back cross populations were developed by Plant & Food Research Institute. To ensure the presence of both transgenes, standard PCR using seed genomic DNA as template was carried out to identify the double transgenic lines. Developing leaves and seeds from the parental lines and identified double transgenic lines provided the material for the gene expression to be performed in Chapters 3-5.

#### 2.1 Identification of double transgenic lines

To identify double transgenic seed lines, a total of 80 seed lines of the transgenic back cross 4 (BC4) population, developed by Plant & Food Research Institute, were tested. In each seed line, DNA was isolated from cotyledons of 11 seeds. If all 11 samples showed positive result for both donor and recurrent parent, another 11 seeds were tested to confirm for planting and they were considered as double transgenic lines.

There were 7 different transgenic BC4 families with different donor parent and recurrent parent. They are MT11/MT1, MT11/MT2, MT2/MT11, MT11/MT3, MT3/MT11, MT11/MT4, and MT4/MT11. In this work, the seed lines containing MT11, MT1 and MT2 were studied.

- MT11 gene constructs contains - AtAAP1:: PsSUT1
- MT1 gene constructs contains - 35S:: PsAAP1
- MT2 gene constructs contains - AtAAP1:: PsAAP1
- MT3 gene constructs contains - 35S:: ScMMP1

- MT4 gene constructs contains - AtAAP1:: ScMMP1

### **2.1.1 Sample preparation and DNA extraction**

DNA was extracted for PCR from dry seeds of pea (*Pisum sativum*) using a rapid method developed by Thomson and Henry (1995). DNA from 11 dry seeds of each line were extracted at a time.

The seed coat was partly removed by using a sharp scalpel. 5-10 mg of tissue was scraped from the cotyledon using the scalpel and was put into the well of a 96 well PCR plate. 30 µl of TPS buffer (Appendix 5.1.1) was dispensed into each well and the plate was spun briefly to mix the plant tissue into the liquid. The plate was then heated to 96°C for 10 min in a PCR machine. After heating, the plate was centrifuged at 4000 g for 3 min and the supernatant was transferred to a fresh plate and stored at 4°C. This was used as the PCR template for testing the gene of interest.

### **2.1.2 Polymerase Chain Reaction (PCR)**

1. The PCR reaction mix (15 µl) contains:

- a) 1.8 µl of 10X PCR buffer
- b) 2.1 µl of 25 mM MgCl<sub>2</sub>
- c) 0.24 µl of dNTP mixture (2mM)
- d) 0.15 µl of primers (20µM)
- e) 0.15 µl of Taq polymerase (5U/µL)
- f) 9.41 µl of double distilled H<sub>2</sub>O
- g) 1 µl of template DNA

2. PCR conditions

- a) 94°C for 3 min
- b) 94°C for 30 s
- c) 52°C for 30 s
- d) 72°C for 60 s
- e) Repeat steps b to d for 35 cycles
- f) 72°C for 7 min

### **2.1.3 Agarose gel Electrophoresis**

The PCR products were assessed by running the DNA through 1% (w/v) agarose gel. The gels were made by mixing the appropriate weight of agarose in 1x TAE buffer (Appendix

5.1.2) and heating in a microwave until all the agarose had dissolved. Then 5µl of ethidium bromide (EtBr) dye per 100 ml agarose was added and swirled to mix. Once the solution had cooled to approximately 60°C, it was poured into a casting unit with four 26-well combs and allowed to set. Samples (5µl) were loaded into the wells. Bioline HyperLadder 1 (4µl) was used for measuring the size and molecular weight of bands. Gels were run at 100 V for about 15-20 min using 1x TAE buffer as the tank buffer. Gels were visualised using a Gel Documentation System (SynGene Bioimaging) and recorded.

## **2.2 Planting**

Eight sets of double transgenic lines were identified, from which three sets of seed lines were planted along with five different control lines in the Biotron at Lincoln. The transgenic seed lines planted were: 104/55 562; 104/55 242; 104/55 382 which contains MT11 and MT1 genes, 104/2/58 8298 that contains MT11 and MT2 genes and 2/58/104 4241, 2/58/104 5156 which contains MT2 and MT11 genes respectively (Table 2.1). The control lines were MT11 (104) single transgene parental line, MT1 (55) single transgene parental line, MT2 (2/58) single transgene parental line, and wild type Bolero and Bohatyr. Twenty-two seeds of each line were sown (planted) on the 23<sup>rd</sup> December 2011. Each seed was sown in a pot containing potting mix, mixed with fertilizers. Growth room conditions, used for the plants are 22°C for a minimum 16-h day and 14°C for an 8-h night. Plants were irrigated every two days, to keep the plants moist but not wet. Plants were fertilized two or three times during the growing period.

Table 2.1: List of transgenes and the seed lines studied in this thesis

<b>Lines Recurrent parent/donor parent</b>	<b>Transgenes</b>	<b>Lines tested/no. of double homozygotes</b>	<b>Line number used in the thesis</b>
Bolero			
MT11	AtAAP1::PsSUT1		104
M11/MT1	AtAAP1::PsSUT1 / 35S::PsAAP1	19/4	242,382,562
MT11/MT2	AtAAP1::PsSUT1 / AtAAP1::PsAAP1	14/3	8298
Bohatyr			
MT2	AtAAP1::PsAAP1		2/58
MT1	35S::PsAAP1		55 or 1/55
MT2/MT11	AtAAP1::PsAAP1 / AtAAP1::PsSUT1	11/3	5156

## 2.2.1 Confirming transgenic lines

When seedlings started to grow, after about two weeks, a small amount of leaf material was taken from all the transgenic plants and extracted DNA for PCR confirmation of double transgenic lines. 10-20 mg of leaf tissue was taken from the growing plant using a forceps and was put into the well of a 96 well PCR plate. 30 µl of TPS buffer (Appendix 5.1.1) was dispensed into each well and the plate was spun briefly to mix the plant tissue into the liquid. The plate was then heated to 96°C for 10 min in a PCR machine. After heating, the plate was centrifuged at 4000 g for 3 min and the supernatant was transferred to a fresh plate and stored at 4°C. This was used as the template for PCR.

## 2.2.2 Labelling and sampling

### 2.2.2.1 Leaf samples:

The leaf samples used in this thesis were developing leaves and mature leaves. The leaf samples were collected at time of sampling of pods. Different developmental stages of leaves were collected as samples. Leaves supporting the pods of 5, 10, 15, 20, 25 DAF and senescence leaves were collected. For the analysis, leaves of two different developmental stages, that is, 5 and 20d were chosen. Both types of leaves were fully expanded but those associated with pods 5 DAF are here referred to as “young” and those collected when pods were 20 DAF are referred to as “older” source leaves. These samples were collected into 15 mL falcon tubes and were frozen in liquid nitrogen and stored at -80°C.

### ***2.2.2.2 Developing seeds:***

When flowering had started after five to six weeks of growth, flowers were labelled with date and flower number using tags. Fifty flowers were tagged in each seed line. Flower tagging was done in such a way to ensure enough samples ranging from 10 day after flowering (DAF) to 37 DAF to be collected. Exactly ten days after flowering, harvesting of these labelled pods started. The pods were collected from 10, 12, 14, 16, 18, 20, 23, 30, 37 DAF from each seed line. Developmental stages were estimated based on the relative water content of 85%- 30% (12-37 DAF). When %DM and DAF were compared, it was calculated that 10% of DM was equivalent to 10 DAF. At 12 DAF, the DM% was 15, on 14, 16, 18, 20, 23 and 30 DAF the DM% was 16-20%, 18-25%, 22-30%, 28-38%, 35-40%, 40-50% respectively.

The pods were harvested directly on to ice and then dissected in a clean petri dish using forceps and scalpel. Each pod was dissected to take 4-5 seeds from it. Each seed was divided into 4 different parts. Each division was stored in respective labelled Eppendorf tubes and was used for different molecular and biochemical analyses. The tubes for biochemical analyses were pre-weighed and were also weighed with samples. For each stage of development, five pods were collected from all seed lines which means 45 pods were sampled for one particular line and a total of around 500 pods were collected. Hence, different tissue samples were collected and stored while dissecting a pod. The separated pod walls were collected in 30 mL falcon tubes. For each stage, five pod walls were collected.

Meanwhile, embryo and seed coat were removed from each seed and collected into different tubes for gene expression analysis. For each pod, five to six seed coats were collected in a tube and were stored at -80°C.

For the research in this thesis, early developmental stages of pod and seed development were also of interest. Developing pods were collected from the days -1, 0, 1, 3, 5, 7 DAF for gene expression studies. During the flowering stage, flowers were date-labelled using tags according to when the flowers opened. The day of full bloom was recorded as 0 DAF. Petals and sepals were removed from the -1, 0, 1, 3 DAF flowers, so that only the tiny ovules remained. The seeds were able to be separated from pod walls at stages 5 and 7 DAF.

- For -1, 0, 1 DAF - 5 to 10 pods per seed line (around 55-100 samples)
- For 3 DAF - 40 pods per seed line (around 440 samples)
- For 5 DAF - 20 pods per seed line (around 220 samples)
- For 7 DAF - 12 pods per seed line (around 132 samples)



All samples were frozen in liquid nitrogen as soon as they were picked off the plant and stored at -80°C.

## **2.3 Gene expression analysis**

### **2.3.1 Sample preparation**

Various tissue types of different developmental stages were studied in terms of gene expression. Early developmental stages included pods of -1, 0, 1, 3, 5, 7 and 10 DAF. Seeds were separated from pod walls at 5, 7 and 10 DAF. Later stages of seed development included cotyledons of 12, 14, 16, 18, 20, 23, 30 DAF. Seed coats separated from seeds of 10 to 30 DAF were also analysed. Additionally, two different stages of leaves were chosen to represent sink and source leaves.

The tissue was ground into a fine homogeneous powder using a mortar and pestle, and kept frozen using liquid nitrogen. Prior to use, the mortars and pestles were thoroughly washed and then decontaminated by baking in a hot air oven at 400°C for 4-5 h. Liquid nitrogen was used to grind the samples to minimise degradation of RNA. The ground samples were then transferred to 1.7 mL Eppendorf tubes and stored at -80°C.

### **2.3.2 RNA extraction**

Total RNA was extracted using TRIzol® by a phenol-chloroform based extraction method: 1mL of TRIzol® reagent (Ambion) was added to about 100 mg of ground frozen plant tissue in a 1.7 mL Eppendorf tube and the material mixed well into the liquid by using a vortex mixer and incubated at room temperature for 5 min. The tubes were then centrifuged for 2 min at 12000 g at 4°C. The supernatant was transferred to a new tube and 200 µl of chloroform was added and the mixture was shaken vigorously for 15 s, incubated at room temperature for 10 min and then centrifuged at 12000 g for 15 min at 4°C. The top layer of the supernatant was transferred to a new tube and allowed to stand for 5 min at room temperature, after which the RNA was precipitated with 500 µl isopropanol, by mixing gently and incubating at room temperature for 10 min. The tubes were then centrifuged at 12000 g for 5 min at 4°C to pellet the RNA. The supernatant was discarded and the RNA pellet was washed twice with 1 mL 75% (v/v) ethanol, centrifuged at 12000 g at 4°C for 2 min, and any

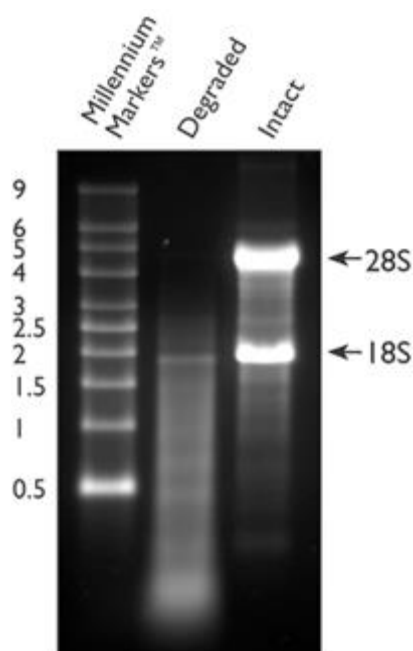
remaining ethanol was aspirated and the pellet air dried for 12 min. The RNA pellet was dissolved in 30-40 µl of 1X RNA Secure (Ambion) and then incubated at 60°C for 10 min to deactivate any RNase. The RNA was stored at -20°C prior to cDNA synthesis.

### **2.3.3 Quality and quantity check of RNA**

The integrity and size of the extracted RNA were assessed by running the RNA through a 1% (w/v) agarose gel and its concentration and purity was assessed using Nanodrop™ spectrophotometry.

#### ***2.3.3.1 Agarose Gel Electrophoresis***

The gels were made by mixing the appropriate weight of agarose in 1x TAE buffer and heating in the microwave until all the agarose had dissolved. Once the solution had cooled to approximately 60°C, 2 µl of SYBR Safe™ DNA Gel Stain per 30 ml agarose was added and swirled to mix. It was then poured into a gel casting tray with the appropriate comb and allowed to set. 2 µl of each RNA sample was loaded into wells with 1 µl 6x agarose-gel loading dye and 3 µl tank buffer. Bioline HyperLadder 1 (2 µl) was used for quantifying the size and of bands. Gels were run at 80 V for about 40 min. Gels were visualised using a Chemi Genius2 BioImaging System (SynGene) and recorded. The integrity of RNA was assessed by the structure and distribution of 18S and 28S rRNA bands. As the 28S band should be approximately twice as the intense as the 18S band, the ratio of 2:1 is denoted as good RNA without degradation.



**Figure 2.1 RNA gel showing intact and degraded RNA** along with Millenium markers™, (www.ambion.com).

#### **2.3.3.2 Nanodrop™ Spectrophotometry**

A NanoDrop® (ND-1000) spectrophotometer (Thermo Fisher Scientific Inc.) was used to measure the quantity and purity of RNA samples. The NanoDrop utilises the absorbance peak of RNA (260 nm) to calculate concentrations and provides an estimate of purity by assessing the 260/280 nm and 260/230 nm ratios. Samples with 260/280 and 260/230 ratios of above 1.8 and 1.5, respectively, are generally accepted as good purity for gene expression studies.

#### **2.3.4 cDNA synthesis**

Synthesis of cDNA from RNA was done by using reverse transcription. The amount of RNA used for cDNA synthesis was 0.5-1 µg, the volume was varied depending on its level of degradation, purity and concentration, but generally between 1 and 4 µl of RNA was used. The template RNA was then added to the primer annealing mix (Appendix 5.1.8) which made the total volume of 10 µl, in a sterile RNase free Eppendorf tube. This was then heated to 65°C for 10 min and then transferred immediately onto ice.

The reverse transcriptase mix (Appendix 5.1.9) was added to the RNA-primer mix in a PCR tube, which made up the final volume of 20 µl. This was then put in the PCR machine (MultiGene Gradient, Labnet International) for a standard reaction of 25°C for 10 min, 42°C for 60 min, 75°C for 10 min, and cool down to 4°C. The obtained product was diluted 10-fold using millipore H<sub>2</sub>O and stored at -20°C.

### 2.3.5 Primer design

Prior to designing primers for the multi-gene family members of *PsIPT*, *PsCKX*, *PsSUT*, *PsAAP*, *PsINV* and *PsSW*, a pea transcriptome analysis was done by Dr Jiancheng Song. Material for the transcriptome was from pea plants grown for 60 days and collecting plant tissues, consisted of pods, flowers, ovules, seeds and leaves. Primers were designed by Dr Song using the Primer Premier 6.0 software and are listed in following tables.

#### *PsIPT* primers:

Table 2.2: List of primers designed for different *PsIPT* gene family members. The bold ones were used for gene expression analysis.

Primer	Sequence
<b><i>PsI604</i> F1:</b>	<b>GCATGATTGAAGCGGGTCAAGTTAAC</b>
<b><i>PsI604</i> R1:</b>	<b>TGAACTTGTTTACTTGCAAGGTTACAG</b>
<i>PsI604</i> F2:	GGGTCAAGTTAACGAAGTTCGTGAC
<i>PsI604</i> R2:	CATCAAGACGGTGCATGTTCCCTC
<b><i>PsI605</i> F1:</b>	<b>GGGTTGATGTTGCACTTCCTGTC</b>
<b><i>PsI605</i> R1:</b>	<b>ATCGGCTGAATTTGCTTCTGCAG</b>
<i>PsI605</i> F2:	TCTTCGCTTCAATCGCGTGTTG
<i>PsI605</i> R2:	TGTCGTTGTCGATTTGCAAGAGTG
<b><i>PsI421</i> F1:</b>	<b>GCTGAAACGAGTCGACGACATGT</b>
<b><i>PsI421</i> R1:</b>	<b>CCTTCACTGCTTCCTGGTATGCAC</b>
<i>PsI421</i> F2:	GAAACGAGTCGACGACATGTTTGAC
<i>PsI421</i> R2:	AGCTGACACGTGTTATCCTTAATCG
<i>PsI424</i> F1:	AAAGAGAATGTAGTTTGGTGATGGGAG
<i>PsI424</i> F2:	TGATGGGAGCAACAGCCACAG
<i>PsI424</i> R1:	TGTGAACTCTATGTTGGGGTTGTGT

[Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]

## ***PsCKX* primers:**

Table 2.3: List of primers designed for different *PsCKX* gene family members. The bold ones were used for gene expression analysis.

Primer	Sequence
<b><i>PsC910</i> F1:</b>	<b>GGGATGATAGACACTCAGTAGTGGTAC</b>
<b><i>PsC910</i> R1:</b>	<b>TCTCTGAACAAATCTATTCCATTTATCTCCA</b>
<i>PsC910</i> F2:	CACCAAAGGGACCACCTACTGA
<i>PsC910</i> R2:	CAGGTGCAAGAATAGCCATTGGATC
<i>PsC910</i> RF:	GCTATTTAAAGCCCAACCACACCTG
<i>PsC910</i> RR:	ATATGAATGCATACACCTTAGTAATTGGTTG
<b><i>Ps942</i> F1:</b>	<b>TGTTTTACCTGGTGGCATTCTGAG</b>
<b><i>Ps942</i> R1:</b>	<b>GAATTGCATCTTCATGGCATTGAATTG</b>
<i>Ps942</i> F2:	TTGGATGCTGAGACACTGGAGTAC
<i>Ps942</i> R2:	CGCTGGCCTGTTGCTAAGATTC
<i>Ps942</i> RFa:	AAGAAACAATGGCGGCAGTGAAG
<i>Ps942</i> RFb:	AGGGCACGGACACTCGATAAAC
<i>Ps942</i> RR:	GTTTAAGCTCTAATCTCACTGCAAAATAATTATTC
<i>PsC209</i> F1:	AAATGTATCATTTGTTGAGTTCTTGAATAGAGT
<i>PsC209</i> F2:	TTGAATAGAGTTAGAAGTGGAGAGTTGAAG
<i>PsC209</i> R1:	TCATCTGGTATTGTTGCTGACATC
<i>PsC209</i> R2:	CTTTCCAATTATCAAATCTACTTGAGTGCA
<i>PsC209</i> RF:	TCCCACAAACACTACACAAAACCAC
<i>PsC209</i> RR:	AATGATTCTGTCAACCTAACATATCTAAGGT
<i>PsC399</i> F1:	ATCACTAAAACAACATGAAATGCAGGTAC
<i>PsC399</i> F2:	GTACATGTTGGGAACCTTCCTCCATAC
<i>PsC399</i> R1:	TGAGGACCATGCTTAAACGCTTGT
<i>PsC399</i> R2:	CCCTGTTCTTATAACAATCTGAAGCTG
<i>PsC399</i> F3:	AGAGACCAAGAGAGATTAATATCTCAAGATAATG
<i>PsC399</i> R3:	GTTGATGTATTTAGCTAATTCTAGGCAGAAG
<i>PsC399</i> RF:	TCATACGCATTCTCATAGGAATAGTTGA
<b><i>PsC627</i> F1:</b>	<b>TTTACAGTGTACTTGGAGGCTTAGGA</b>
<b><i>PsC627</i> R1:</b>	<b>AGTTCTTCCATCTGATTTGAACCTTGCT</b>
<i>PsC627</i> F2:	AGTTGAGAAACACTTGTCCTTCTTG
<i>PsC627</i> R2:	GATAGGACCATTGCTTGTTTCTTTGAC
<i>PsC627</i> RF:	TGGCTTTAACCTTCTTCACCCTTTAAC
<b><i>PsC930</i> F1:</b>	<b>GGTCCCATACTCATTTACCCTGTCA</b>
<b><i>PsC930</i> R1:</b>	<b>GGCATGGGTGCAGAAATCTAAGATC</b>
<i>PsC930</i> F2:	ACCAGGTGGAACCGTAAAACATCT
<i>PsC930</i> R2:	GTAATGAGCAAGATATTGCTTCACATTTAGT
<i>PsC930</i> RFa:	GGTGAAATGGATTAGGGTGCTCTATTC
<i>PsC930</i> RFb:	GAGATCAAGAAAATTTGATATCACTTAAGGACA

PsC131 F1:	YATCTCNNNYTTGATAAAEWTTCTCAAAYTCTC
PsC131 F2:	ATGGGGTKGTGKTGAAYATGACT
PsC131 R1:	GTAACAACATCCAATTCAWGAACATTGG
PsC131 R2:	TTCTCCKYAGARCAAGTCACWAKG

[Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]

### ***PsSUT* primers:**

Table 2.4: List of primers designed for different PsSUT gene family members. The bold ones were used for gene expression analysis.

Primer name	Sequences
<b>PsSUT366F</b>	<b>5 TGGGCAGTTATCCGGTGCTTTC</b>
<b>PsSUT366R1</b>	<b>5 ACACCCATATCATACGCATGACCTTC</b>
PsSUT366R2	5 GCCAAGATATCAACACCCAGTGAC
<b>PsSUT948F</b>	<b>5 CAGTGCTTCAGGGGCTGGAC</b>
PsSUT948Ra	5 CAACACTATCGCCAATACAGCACTG
<b>PsSUT948Rb</b>	<b>5 GTAAAATAATAATTGCTAATACACCACTGATGAC</b>
<b>PsSUT666F</b>	<b>5 CCGATCGTCGGTTACTACAGTGATC</b>
<b>PsSUT666R1</b>	<b>5 CCCCAGTGAAGGCACGACATG</b>
PsSUT666R2	5 CTTCTATGGTCGTCAGCAGCGAG
PsSUT164F	5 GGGAAGTGGACCATGGGATCAG
PsSUT164R1a	5 CATATGATACCTCATAACGATTTCGAG
PsSUT164R1b	5 GAACATGATACCTCATACTGGGTTTCTAG
PsSUT164R2a:	5 GGTTGAAATCTTGAGGAGAGGAAAACCTC
PsSUT164R2b	5 CCACAGCTAAAGTGAGGTTGGATG
<b>PsSUT674F</b>	<b>5 AATGTGTAAGTGGATGGGTGCAAG</b>
<b>PsSUT674R1</b>	<b>5 AAKGGAACACTRTAGGTAATCGCAAG</b>
PsSUT674R2	5 ACCWCCTGAATCAGCAGTYAAYTCTG
<b>PsSUT102F</b>	<b>5 AGGGATCATTACCAAAGGCGAAG</b>
<b>PsSUT102R1</b>	<b>5 GTWAAGARRACTTGAGCAATGATAAAKGTG</b>
PsSUT102R2	5 CCACAMYCTATTCATTCTTCAATTGGAAC
PsSUT428F	5 CATTACCAAGGCAGCTCAGCATG
PsSUT428R	5 GARCTCCSARAGATAAACCTTGTCAG

Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]

## PsAAP primers

Table 2.5: List of primers designed for different PsAAP gene family members. The bold ones were used for gene expression analysis.

Primer name	Sequences
<b>PsaAP498F</b>	<b>5 AGGAGTGTGTCTTTGGCATGGAG</b>
<b>PsaAP498R</b>	<b>5 ATGGATGCTRTAATGATGTAGGCAATTC</b>
PsaAP600F	5 CWTTTCAGAACTGCTTATGTTGCATCAAC
PsaAP600R	5 YYAATGGATCCAACMAGAGTGAACAATC
<b>PsaAP9261F</b>	<b>5 ATTCATTTGGTTGGAGGATATCAGATATACAG</b>
<b>PsaAP9261R</b>	<b>5' TCCAGTGGTTGAAATCACATAGGTTG</b>
<b>PsaAP9262F</b>	<b>5 ATTCATTTGGTTGGAGGATATCAGATTTATAG</b>
<b>PsaAP9262R</b>	<b>5 TCCAGTTGTGGAAATCACATAAGCTG</b>
<b>PsaAP446F</b>	<b>5 TGCAATTGGTCTTTGTCTTTCCGTAC</b>
<b>PsaAP446R</b>	<b>5 TTTCTGATGGAGGTGACCTTAGTGTATC</b>
PsaAP303F	5 AGGACAAGTTATTGGAGTGGATGTCAC
PsaAP303R	5 CACACATTGCATAGAAGAATGTGGTGATTG
PsaAP367F	5 CGGGCTCGTTGTTGGAGTGGATGTCAC
PsaAP367R	5 CGCATAATGAATAAAAGAATGTGGTGATTG
<b>PsaAP051F</b>	<b>5 CCGGTTTCGGTTTCTATAATCCTTTCTG</b>
<b>PsaAP051R</b>	<b>5 RWTGACCTCCAAACCAATCTAAAGAGGTTG</b>
<b>PsaAP224F</b>	<b>5 CGGAAGTGTTTGGACTGCAAGTTC</b>
<b>PsaAP224R</b>	<b>5 TACTCCACCAAGAATGGAGCGAAC</b>
<b>PsaAP675 F</b>	<b>5AGGTACGGTTTGGACAACGAGT</b>
<b>PsaAP675 R</b>	<b>5 GAGAATGGTGTGAACAGCTTCCATG</b>
<b>PsaAP328F</b>	<b>5 AGAAGTTGTAAGATCTGTCTTAGGAGGAAG</b>
<b>PsaAP328R</b>	<b>5 GCTGCAACTATTGAGAGCCATGAAAG</b>
<b>PsaAP931F</b>	<b>5AAGCTTTGTGCATTGGCTCAGTAC</b>
<b>PsaAP931R</b>	<b>5 GCAGCAATAATAGAGAGCCATGAAAGTTC</b>
<b>PsaAP532F</b>	<b>5 GGATACACGGTAGCTTCTGCCATAAG</b>
<b>PsaAP532R</b>	<b>5 GCCTAGACCAATCGTCGAATATGTAAAG</b>
PsaAP180F1	5 GGACTTTCTCTTCCACCGTTATTCAAG
PsaAP180R1	5 TGGAGGTGATGATTTTCAGAGTGTCTTG
<b>PsaAP180F2</b>	<b>5 ACTATCAATTATTTTAGGCTACTTTGGAGGAC</b>
<b>PsaAP180R2</b>	<b>5 CTCATGTTTGAAGCCCAATCCAAC</b>
<b>PsaAP840F</b>	<b>5 TGTAGGATTTCAAGCAATCCATACATGATC</b>
<b>PsaAP840R</b>	<b>5 TGTTGATGACACATTTCCACTGCTTC</b>
<b>PsaAP4401F</b>	<b>5 CATGGGTAGCCTCACAGGAATCAG</b>
<b>PsaAP4401R</b>	<b>5 CAAAGCATGTAAAATGTTGTGGTCACTG</b>
<b>PsaAP4403F</b>	<b>5 CGCTGGATTGTTGTCTCGAAAG</b>
<b>PsaAP4403R</b>	<b>5 CATAAATATGTTTGTATTGTAGACTGGCAAAC</b>
<b>PsaAP4405F</b>	<b>5 GCTATGCTGCATTTGGAGACACATC</b>
<b>PsaAP4405R</b>	<b>5 CCAAATTAATCGAAATAGATTTAGATGGTAAGGATG</b>

[Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]

## ***PsINV* primers:**

Table 2.6: List of primers designed for different *PsINV* gene family members. The bold ones were used for gene expression analysis.

Primer	Sequences
PsINV448F1	CAATACAACCCTAAAGGTGCACAATG
<b>PsINV448F</b>	<b>AGGTGCACAATGGGGAAATATAGTATG</b>
<b>PsINV448R</b>	<b>GATCCATTCCCTTAGAAAAGGGTCAG</b>
PsINV448R2	CTGGTGACTTGATCCATTCCCTTAG
<b>PsINV681F1</b>	<b>YCATATTGAAGCTTTCCATCAAACCTCATTC</b>
PsINV681F2	GCCTTCTTTTCACTTCCAACCTTC
<b>PsINV681R1</b>	<b>GCATGTTGTAATGAGAACCAATTCACTAG</b>
PsINV681R2	TGAACCTGACCAACAACCATTGATG
PsINV327F1	CGGTGAAGGAAATTGAAAATCTACGTATG
PsINV327F2	TGATTAAAGGAGGTGAAAGGATTTCATTAC
PsINV327R1	CCAACGCCACCTTTTACACCTG
PsINV327R2	ACTGCTGTATATTCTTTCAACCTTGAG
<b>PsINV285F1</b>	<b>GGRAARCAATTGRTYCAATGGCCTATAG</b>
PsINV285R1	GGGTYTAAKRCTTGTGCCTTTTCAAG
<b>PsINV285R2</b>	<b>GYTGAGGGTCCAWTTTCCAACCTAG</b>
<b>PsINV415F</b>	<b>ACCAAAGCAGGTCTTCTTTGAATCAC</b>
PsINV415R1	GTGTGCGTTATCATTGATTGCCAAC
<b>PsINV415R2</b>	<b>GCACTTAAYTTCTYGATCTTAACATCAACTG</b>
<b>PsINV320F1</b>	<b>TGTAGCAAACMATATGCATCAAGAAATG</b>
PsINV320F2	GGGCCATTTGGTTTGTAGCTTTAG
<b>PsINV320R1</b>	<b>AATAATGGACTTGTCAATCAAGCTTCTAAG</b>
PsINV320R2	CATCTCCAAAACCTCTCAATAATGGACTTG
PsINV885F	STTGAGRATRRTGGTTGGTAGTAGAAG
PsINV885R1	CATGAYTACCTTCAATTGATGTATCCAAC
PsINV885R2	AATGTRTAGTACTCAAATCTAGTCATRTCAAGAC
PsINV376F1	AAGCTYYAAASAAACATGTRGTTCTCTTATG
PsINV376R1	AGCWSWGGATAAACCCCTAGACAAAATG
PsINV376R2	CCMTTGTTTRAAMACAAACAAATGAGCTTG
<b>PsINV240F1</b>	<b>TCGTTGACAAGCGAAGCAATGAG</b>
PsINV240F2	TGCGTCTGATCCGCTTCTCAC
<b>PsINV240R1</b>	<b>CACCAACAAGTATCCTCCACTGTC</b>
PsINV240R2	GAAGTTCCTACTCCTATATAAATAGGCTACTC

[Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]



***PsSWEET* primers:**

Table 2.7: List of primers designed for different *PsSWEET* gene family members. The bold ones were used for gene expression analysis.

Primers	Sequence
<b>PsSW17F1:</b>	<b>TTTAGCAGCAGCAGTAGTAGCAACTC</b>
PsSW17F2:	AAGGAGAAGCTCGTAGTGGTGCTG
<b>PsSW17R1:</b>	<b>GTATCCCAAGAATAATATCACGAACAAGAACAG</b>
PsSW17R2:	ATACAACACCAACTGCATAGCTCCAAG
<b>PsSW13bF1:</b>	<b>TTTCTACCATAAACTACTTCTCTTGCTGAATG</b>
PsSW13bF2:	GATTTCGGAGCCATGCTTCTATCAACTC
PsSW13bR2:	GTAGAGCAACATAGTAATCCTTGAGGAGAAG
<b>PsSW13bR1:</b>	<b>GCCATAGAAGAACCACATAACAGCATTG</b>
PsSW9F1:	CATTGTGTCATTCTTGGTGTTCTTAGCTC
PsSW9F2:	TTCTTAGCTCCATTGCCAACCTTCTATTC
PsSW9R1:	TAACCTTCTATAACACATCCAATACAATTGATGGTG
PsSW9R2:	GCAACAAGTATCAAAATCAAAGTTGATATCTTGAG
PsSW13aF1:	GTCGACATTGAGGATGATTGTGTTGATG
<b>PsSW13aF2:</b>	<b>GAAGGACGTATCAAGCTTCTCGGATG</b>
PsSW13aR1:	GGGTAACATAAAATATCTCTGAGAGAAAGACCATAC
<b>PsSW13aR2:</b>	<b>ACAGTGTGATCTGAACTATTCCAAATGTTAAC</b>
PsSW12aF1:	GTGTAGAGTTTATGCCTTTCACTCTTTCAC
PsSW12aF2:	TCACTCTTTCACTTCTCCTCACAATAAGTG
PsSW12aR1:	GTTTACAGGCTTGTTCTTTCTGTATATTCCATAC
PsSW12aR2:	GCAGCTGATTCTATCTCATCTTTGTGTTT
PsSW624F1:	AGAGTGTTGAGTTTATGCCTTTCAATTTGTC
PsSW624F2:	GTGCCATAATGTGGTTTGGTTATGGTC
PsSW624F3:	GGTCTGTTCTCAAGGACATATGCATTG
PsSW624R:	TTGTTCTTCTCCACTTTGCTCTCTACAAC
PsSW623F1:	AGAGTGTTGAGTTTATGCCTTTCAATTTGTC
PsSW623F2:	GTGCGATAATGTGGTTTGGTTATGGTC
PsSW623F3:	CCTTAAAGATATATGCATTGCTCTACCAAATGTG
PsSW623R:	ATCTTCTTCCAATGGAAACACTTCACCAG
<b>PsSW445F1:</b>	<b>TCAATTGGCTTTAGCTTTTGGCATACTAG</b>
PsSW445F2:	GGTAATGTCATTTCTTCATGGTGTACTTG
<b>PsSW445R1:</b>	<b>CAACACATCCAAATGCATTAATGGTAATGAG</b>
PsSW445R2:	TGGTGGCGTAGATTGTGTAGATGATAATG
PsSW534F1:	CAGTCACTACCATATTTGGTAGCATTATTCAG
PsSW534F2:	ATTATTCAGTTGCATGCTTTGGCTATACTATG
PsSW534R1:	GCCACAAATACTTTTATGGCAAATTCCTTG
PsSW534R2:	GGTATAGCAAATTGTGTGACCAAGAAGATC
PsSW1F1:	WCCWTTTGTGTCWCCACACAACATACTAG

PsSW1F2:	MAATGGCACAGGAGCAGTAATTGAAATC
PsSW1R1:	ACYAATCTCATAATTGAGAGAGGWGAACCATAC
PsSW1R2:	ASAGTGAMARGAAGAATGGCATGAACTC
PsSW4F1:	TGAATCATGACTGCAGCAGAAGCAC
PsSW4F2:	TCGAACAGTTGTTGGTATTATAGGAAATATCATCT
PsSW4R1:	CACGTTGTCCGGGTTCCACCATG
PsSW4R2:	CACAGAACCCGAGCCGTTGATG
<b>PsSW2aF1:</b>	<b>GATCTGCTTGTGGTATGGAACCTCCTC</b>
PsSW2aF2:	GGTTACCACTGTTAATTCAATTGGAGCAG
<b>PsSW2aR1:</b>	<b>GACAAACATTCTGGCGCATAGAACTATC</b>
PsSW2aR2:	GAAATCAGAGAAGCACAACTCAAGCATC
PsSW2bF1:	CGTCTTTGCAATCATATTGATTGGAAGTTTG
PsSW2bF2:	CTTTGTTGGGGTTTTGAGTTGTGCATC
PsSW2bR1:	CGTAAACTAAGAAAGAAGTGCTCATTAGGAAG
PsSW2bR2:	GTTCCATATCCCATTTGGCACATAAATGAAG
PsSW6aF:	TCATGGCAAAGGTTATAAAAACTAAGAGTGT
PsSW6aR1:	GTTAGAACAATAACAAGCATATAGAATAAGCTGAAC
PsSW6aR2:	CTAGTTGGTTTCATCTCAACATCACCATC
<b>PsSW6bF1:</b>	<b>GTTAGCTCGCACAACCGTTGGA</b>
PsSW6bF2:	CCGTTGGAATCATAGGGAATGTCATCT
<b>PsSW6bR:</b>	<b>ATGGTATAAACTAAAGTGCTATTTGGATGCAC</b>
PsSW5F1:	GTCTTTCTTGTGTTGGTGATATTTATTGTCATG
PsSW5F2:	AGGAGAGAAGGTTGGTTGTTGGATTTATATG
PsSW5R1:	GTTGTCCAAATAACTCCATTGAGGAAGTTG
PsSW5R2:	GCCATTGGGAACCACTATGAATGGATC
PsSW7F1:	TTTGTGCACCCAGACAGCACTTTAG
PsSW7F2:	GGTGTGAGTTGTGATTGAGATTATCTATC
PsSW7R1:	TTTTGTAGTCCCATGAAGTGCTAACATTG
PsSW7R2:	CATTACATTGAAGACATCACATATAACACCAAC

[Note: N = ATGC, M = AC, R = AG, Y = CT, W = AT, K = GT, S = GC, H = ACT, B = CGT, V = CG, D = AGT]

## **2.3.6 Primer selection/ sequencing**

### ***2.3.6.1 Polymerase Chain Reaction***

Primers were designed for members of each gene family that were detected in the transcriptome and all these were tested through a temperature gradient (52°C, 55°C, 58°C and 60°C) PCR to find the optimal temperature for the primers. Once the standard temperature was selected for each primer set, all possible combinations of forward and reverse primers were tested through standard PCR analyses (55°C and 58°C) to determine whether they reacted with the cDNA samples used in this study. The products were analysed by running them through an agarose gel (1% w/v). Only the primers which gave bright bands were selected for the next phase of testing.

### ***2.3.6.2 PCR product purification***

Following the standard PCR with the selected primer pairs, the size and purity was assessed by running the PCR products through a 2% (w/v) agarose gel. A Safe Imager™ blue-light transilluminator was used to visualise the DNA, the bands were then cut and stored at -20°C prior to purification. DNA was purified from agarose gel by using UltraClean™ 15 DNA Purification Kit. These kits use economical silica binding particles to extract DNA from agarose gels. The desired DNA bands were cut from the gel and melted irreversibly in a chaotropic salt solution. Then the DNA was bound to UltraBIND silica particles in the presence of UltraSALT. The complex was micro-centrifuged to discard the molten gel by leaving the pellet which was washed once in UltraWASH and concentrated by centrifuging and discarding the supernatant. The purified DNA was then eluted in TE buffer. The concentration and quality of the purified DNA was tested by using Nanodrop™ spectrophotometry and agarose gel electrophoresis.

The DNA was sent along with forward/reverse primers to MACROGEN Inc, Korea where the PCR products were sequenced. The sequence verification after sequencing was done for each specific target gene with BLAST (<http://www.ncbi.nlm.gov/BLAST>) searching the

GenBank database and each confirmed target gene was selected and designated to gene family member based on available annotated sequence or putative sequence of the target genes.

### **2.3.7 RT-qPCR analyses**

Relative gene expression was measured using reverse transcription real time quantitative polymerase chain reaction (RT-qPCR). It is a highly sensitive technique which allows quantification of rare transcripts and small changes in gene expression (Pfaffl, 2001). The expression studies were done following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines, written by Bustin et al. (2009).

The real time RT-qPCR assay was validated with optimisation of primers by determining the optimal annealing temperature of all the reference genes and target genes at 52°C, 55°C, 58°C and 60°C. A positive control and a non-template control (NTC) were included in every run to test for DNA contamination and to assess for primer-dimers.

The expression of each gene of interest and selected reference genes were quantified by using Rotor-Gene Q machine (Qiagen, Germany) with the home-made SYBR® Green master mix (Song et al., 2012). This reaction utilises SYBR Green which binds to double stranded DNA and fluoresces allowing the quantification of double stranded DNA. The accumulation of the product is measured in terms of Relative Fluorescence Units (RFU). A reaction volume of 15 µl was used, which consisted of 7.5 µl 2x SYBR® Green dye (Appendix 5.1.10), 1 µl of each forward and reverse primer, 1 µl cDNA and 4.5 µl Millipore H<sub>2</sub>O. The thermal cycle consisted of initial hold at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 58°C for 15 s and 72°C for 20 s. The melting curve analysis was performed at the temperature range of 72°C to 95°C, raising 1 degree each step with a 5 s wait for each step after 90 s of pre-melt conditioning on the first step.

#### **2.3.7.1 Reference genes**

Reference genes were used as internal controls to normalise the data by correcting the differences in quantities of cDNA used as template (Gutierrez et al., 2008).

The reference genes used for all experiments in this study were *PsEF* (ELONGATION FACTOR), *PsGAP* (glyceraldehyde-3-phosphate dehydrogenase) and *PsACT* (Actin). These genes are present in all nucleated cell types, supposed to be expressed at similar levels, since they are necessary for cell survival (Pfaffl, 2001).

Before expression studies for the target genes were done, the concentration of each set of cDNAs was adjusted using the reference genes, with three technical replicates. It was done in such a way that the Ct (threshold cycle) values between each cDNA were within a maximum difference of 1-2 cycles. cDNAs were either diluted or resynthesized to ensure Ct values were within the acceptable range. Ct values for *PsEF* and *PsACT* were generally between 10-15, whereas for *PsGAP* they were between 15-18.

#### **2.3.7.2 Target gene expression analysis**

Expression of genes is presented as relative gene expression based on the method of Song et al. (2012). Relative expression of genes was determined by comparing the expression of target genes to the average expression of three reference genes used. An inter-run calibrator (IRC) was used in each RT- qPCR run, as all the samples could not be tested at one time. The calibrator was the mixture of all cDNAs of all tissue types.

For each reference gene, a correction factor (CF) for each cDNA sample was calculated using the Ct number of this cDNA sample divided by the average Ct number of all cDNA samples included in the same experiment. The values of three to four technical replicates were averaged to form the CF for each biological replicate of each reference gene. The final CF value for each biological replicate was created by averaging the CF values of the four reference genes. For each cDNA sample, the Ct number of each target gene was corrected before statistical analysis of its expression level (Song et al., 2012).

### **2.4 Starch and sugar analysis by enzymatic assay**

The amount of starch and sugar present in the developing seed coats of both field peas (Bohatyr) and process peas (Bolero) was estimated by enzymatic assay by using Total Starch kit.

Prior to the sugar and starch assays, the fresh samples were ground in liquid nitrogen and approximately 100 mg of the ground sample was accurately weighed into a 2 ml Eppendorf tube. Ethanol (80%, v/v) was added to the sample and vortex mixed and incubated at 60°C for 5 min. The well mixed sample was then spun at 14000g for 3 min. The supernatant was then transferred to a 2 ml tube. The supernatant contained the sucrose extract. The extraction was repeated twice, and all the extracts were collected into the same tube. Then the supernatant was dried down in the savant. The pellet containing the starch was kept for the starch assay.

### 2.4.1 Sucrose assay

The assay was based on the amount of NADH produced through a cascade of enzymatic reactions. The amount of NADH formed was stoichiometrically equal to the amount of glucose or fructose present in the solution and was measured as an increase in absorbance at 340 nm (Boehringer et al. 1987). For the calibration curves, a glucose/fructose (G/F) stock solution (10mg/mL of each) was used to make standard solutions containing 0, 0.125, 0.25, 0.375, 0.5, 0.625, 0.75, 0.875 and 1 mg/mL of glucose and fructose. Aliquots (10 µL) of the standards and controls, each in duplicate were dispensed into the designated wells of a 96-well microplate. The samples were dispensed as a block of 24 unknowns each in triplicates of 10 µL. The final sucrose concentration in the sample was calculated as mg/g fresh weight (FW). Sucrose levels in the hydrolysed samples were calculated as:

Sucrose (mg/ml) = total glucose (mg/ml) – free glucose (mg/ml) x 1.9, where

1.9 represents the molecular mass of sucrose divided by the molecular mass of glucose.

Table 2.8 Procedure for Sucrose enzymatic assay

Pipette into wells	Sample fractions	
	Blank sucrose	Sucrose wells
Maltase/Invertase	10 µL	10 µL
Mix, incubate at 40°C for 30 minutes		
Solution 1	250 µL	250 µL
HK/G6PDH	10 µL	10 µL
Mix, incubate at 25°C for 20 minutes and read absorbance at 340 nm		

### **2.4.2 Starch assay**

The starch assay was done from the pellet obtained during sucrose extraction. 20 µl of Ethanol (80%, v/v) and 300 µl MOPS buffer was added to the pellet and was mixed well. The mixture was then incubated in boiling water bath for 12 min, with vortexing every 2 min. The tubes were then transferred to 50°C water bath before acetate buffer was added to it. It was mixed well and added 10 µl amyloglucosidase and kept for incubation at 50°C for 30 min. The tubes were spun at 3000g for 5 min to obtain a clear supernatant. 75 µl of the supernatant was then taken and diluted with 925 µl ddH<sub>2</sub>O. The diluted samples were then dispensed as a block of 24 unknowns each in triplicates of 10 µl into a 96-well microplate with 250 µl GOPOD reagent. Aliquots (10 µl) of the standards and controls, each in duplicate were also dispensed into the designated wells of the plate. The plate was then incubated at 50°C for 20 min and read the absorbance at 510 nm. The final starch concentration in the sample was calculated as mg/g fresh weight (FW).

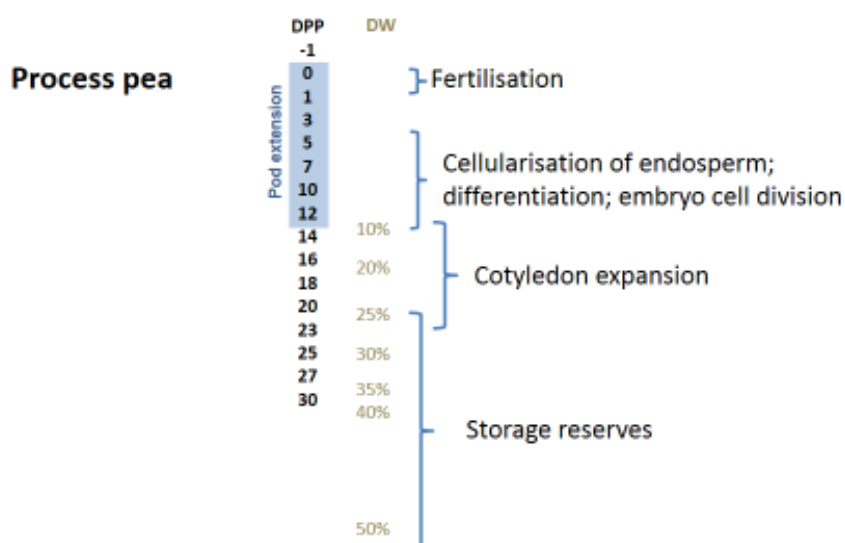
## CHAPTER 3

### Expression of cytokinin and transporter genes in field and process peas

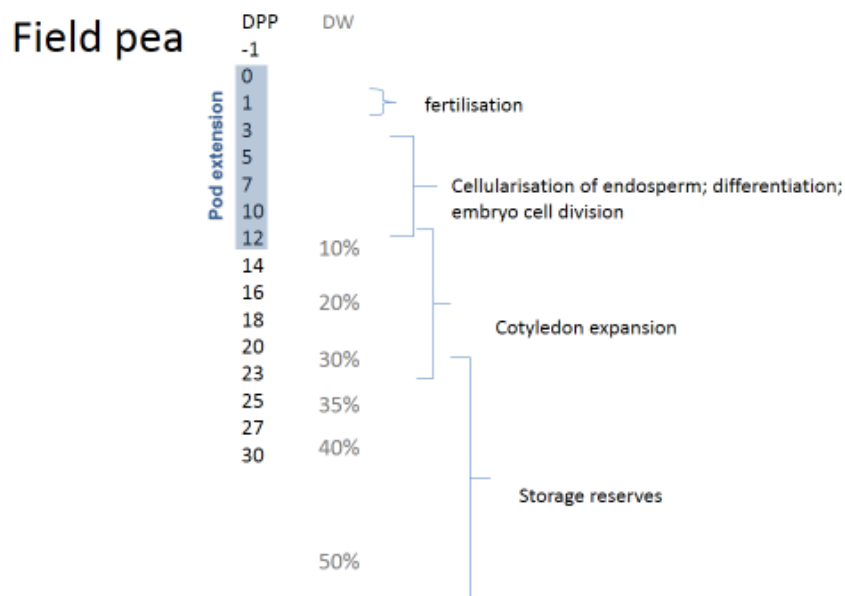
#### 3.1 Introduction

In this chapter, the key nutrient transporter genes and other selected genes were isolated and then expression monitored during seed development in two different cultivars of peas (*Pisum sativum*): a process pea (Bolero) noted for its high sucrose content at time of harvest of the immature seeds, and a field pea (Bohatyr) with high starch content at the time of harvest of mature seed.

Figure 3.1 describes the developmental stages of process pea and field pea. In both cultivars, extension of the pod continues up to 10-12 days after fertilization. The second stage of seed development starts from about 14 days after fertilization when the cotyledons start to expand. In both process pea and field pea, this begins when the dry matter is about 10%. The final stage is the storage phase which starts at around 20 to 23 days after fertilization and the dry weight increases during this phase. When the storage reserves start to accumulate in Bolero, the % dry matter percentage is about 25, whereas in Bohatyr, it is 30-32.



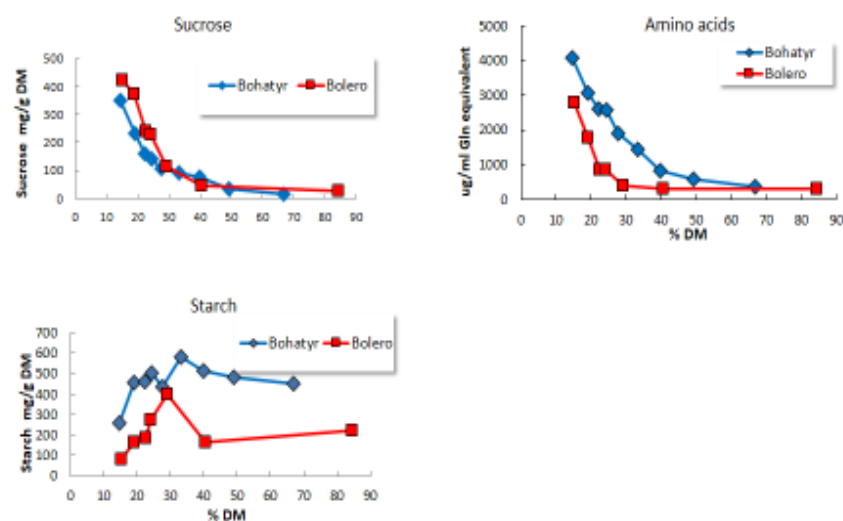




**Figure 3.1: Generalised diagram showing different stages of seed development in process pea and field pea.** DAP means days after pollination, DW means dry weight (Drawn by Jan Grant and Paula Jameson).

The starch/ sugar analyses of the maturing cotyledons was carried out by Plant & Food personnel. Figure 3.2 shows the difference between the field peas and process peas in sucrose, amino acid and starch content of cotyledons during seed development. Sucrose and amino acid content is greatest during the early stages, since cell proliferation requires high amount of metabolic energy. As the seed developed, the sucrose and amino acid content decreased in both cultivars. However, process pea had higher sucrose from 15 to 25% DW, and had a lower amino acid increased in both the cultivars. However, the starch content of field pea increased earlier and is greater at full maturity.

This chapter provides the base-line for transgenic work.



**Figure 3.2: Sucrose, starch and amino acid content in the developing cotyledons.** A and B is sucrose and starch in mg/g of dry matter against % dry matter. C is the amino acid content in  $\mu\text{g/ml}$  of glutamine equivalent against % dry matter. Bohatyr = Field pea, Bolero = Process pea. (The analyses were completed by Plant& Food personnel. Data courtesy of Jan Grant).

## 3.2 Materials and methods

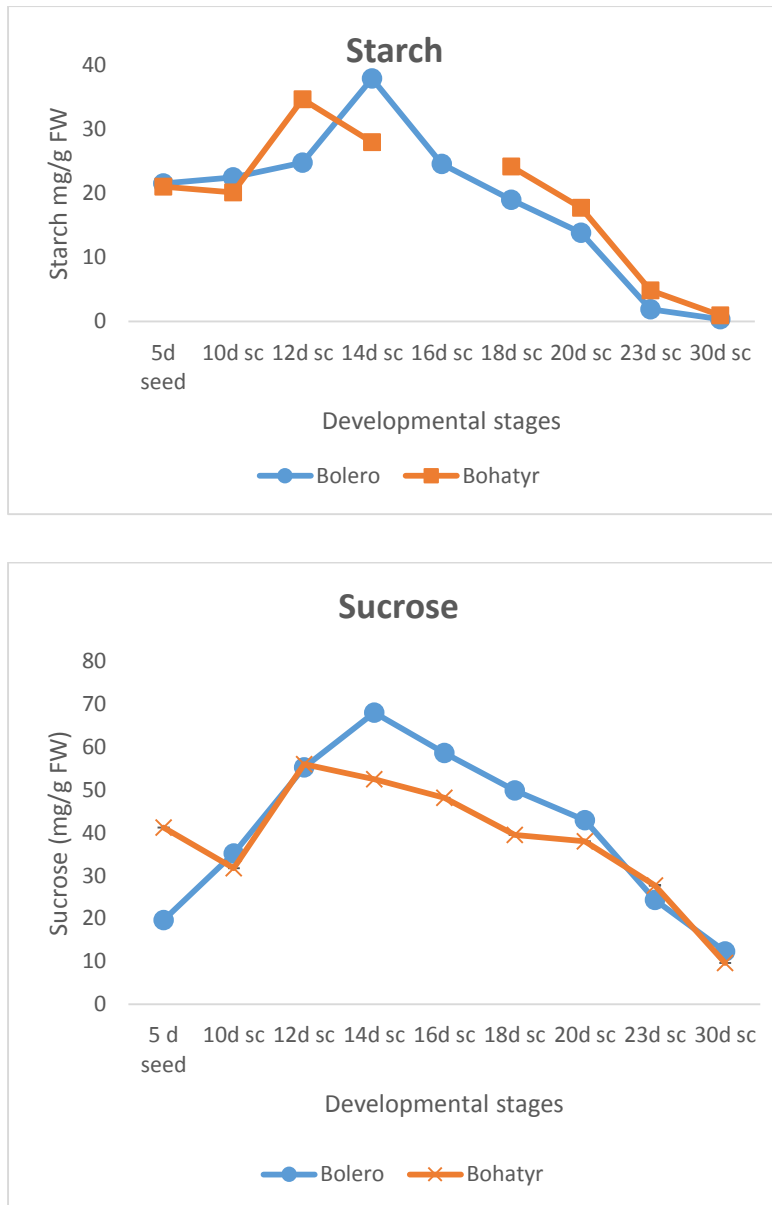
The materials and methods used in this chapter are outlined in Chapter 2. To align with the analyses carried out by Plant & Food Research Institute, the following samples were selected (Table 3.1).

Table 3.1: List of samples at different developmental stages, equivalent to % DM content.

DAF	DM %
10	10
12	15
14	16- 20
16	18- 25
18	22- 30
20	28- 38
23	35- 40
30	40- 50

### 3.3 Results

#### 3.3.1 Starch and sugar analysis of pea seed coat during development



**Figure 3.3: Sucrose and starch content in the seed coats of developing seeds.** Sucrose and starch content was measured as mg/g of fresh weight and was plotted against the developing stages. sc means seed coat.

Figure 3.3 shows the sucrose and starch content in the seed coats of the developing seed of field pea and process pea. The sucrose content is greater in the seed coats of process pea (Bolero) and was maximum at 14 DAF, while that of Bohatyr was at a maximum at 12 DAF. At maturity, the amount of sucrose is much reduced in both Bolero and Bohatyr. But comparatively, Bolero had higher sucrose at 30 DAF.

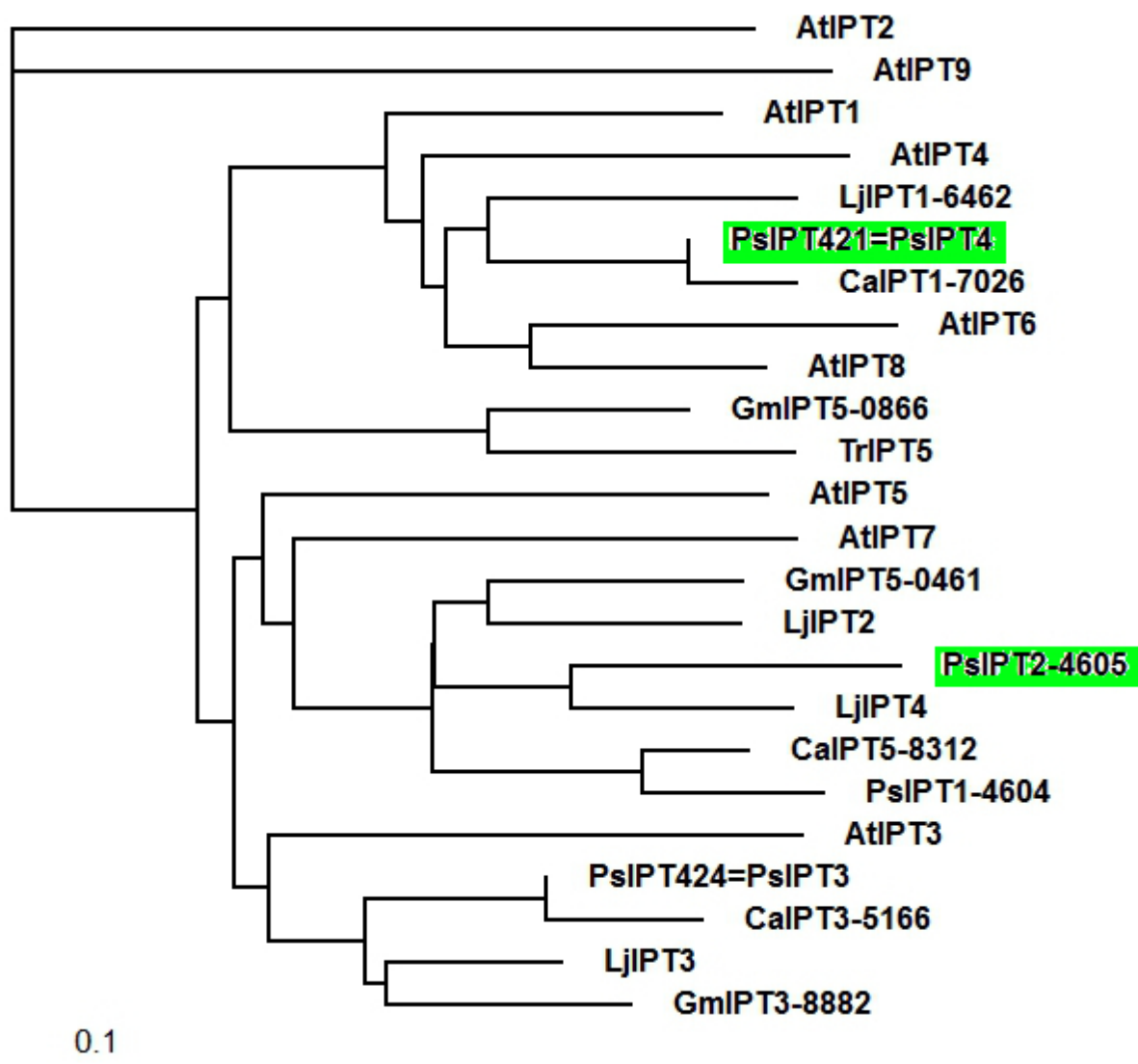
The starch present in the seed coats of field and process peas was highest at 12 and 14 DAF respectively. At the later stages (18, 20, and 23 DAF), the seed coats of Bohatyr contained more starch, compared with Bolero.

### **3.3.2 Phylogenetic analysis**

#### ***3.3.2.1 Cytokinin biosynthetic gene family (IPT)***

IPT primer products were identified and sequenced by Dr Jiancheng Song, based on the pea transcriptome. The primers identified were PsIPT 604F2R2, PsIPT 605 F1R1, PsIPT 605 F2R2 and PsIPT 421 F1R1. From these, PsIPT 605 was verified as PsIPT2, PsIPT 604 was verified as PsIPT1 and PsIPT 421 was confirmed as PsIPT4.

Phylogenetic analysis was done by aligning the sequenced nucleotides, the homologous IPT gene sequences of *A. thaliana* and legumes (*Glycine max*, *Lotus japonicus*, *Pisum sativum*) from BLAST GenBank database. The phylogenetic tree showed three main clades. The isolated PsIPT1 and PsIPT2 were grouped together in one clade along with AtIPT5 and AtIPT7. The isolated PsIPT4 was grouped with AtIPT1, 4, 6 and 8. The AtIPT3 branched with GmIPT3 and LjIPT3 and a putative PsIPT3, although it was not sequenced and did not give any expression for the tissue types studied.



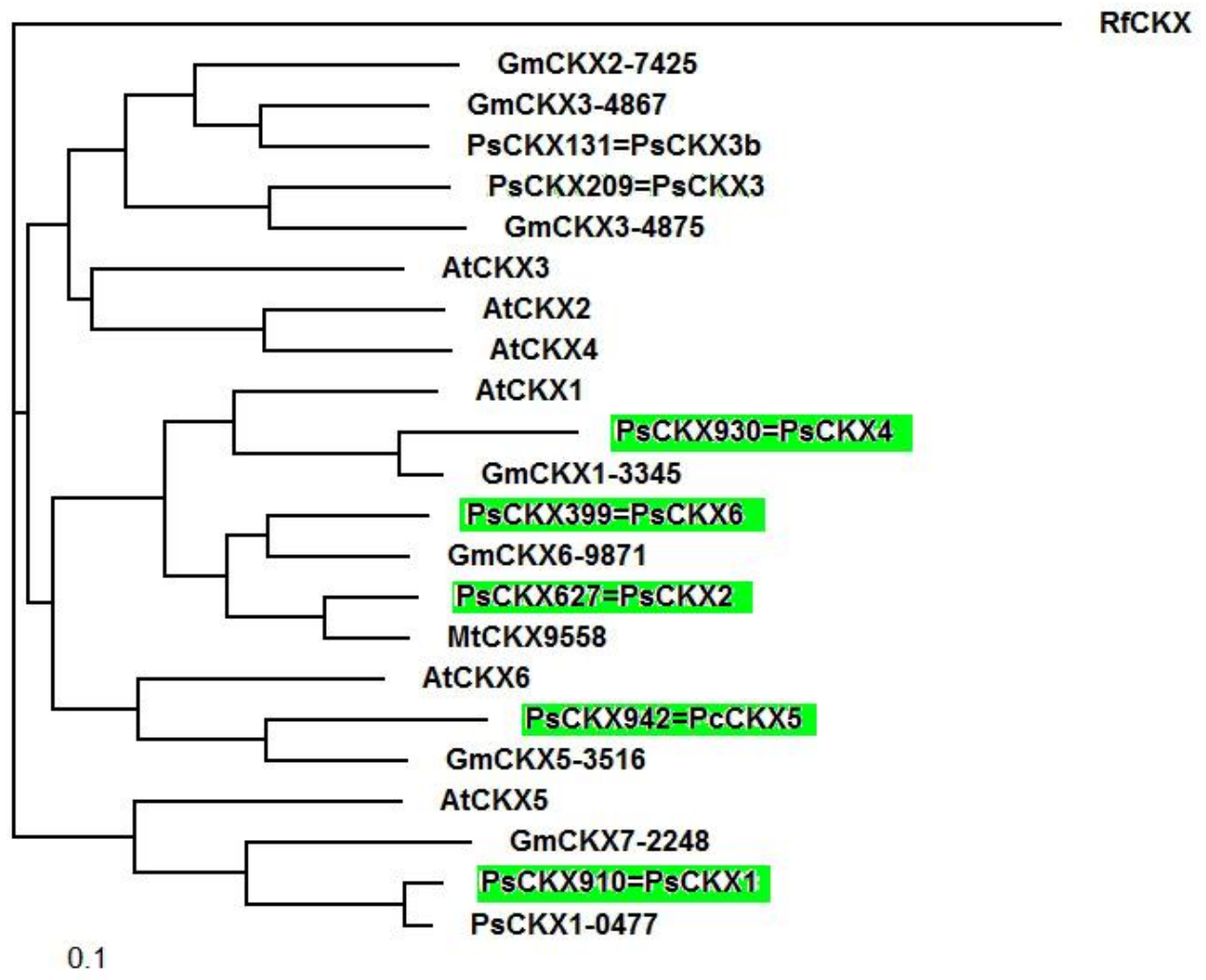
**Figure 3.4: Phylogenetic tree of IPT.** The *PsIPT* gene family members isolated and used in this study are highlighted. The tree was rooted with *AtIPT2* and *AtIPT9*.

### 3.3.2.2 Cytokinin oxidase/ dehydrogenase (CKX) gene family

The CKX primers were designed by using the pea transcriptome. The primers were identified for five different gene family members which are *PsCKX* 910 (*PsCKX1*), *PsCKX* 627 (*PsCKX2*), *PsCKX* 131 (*PsCKX3b*), *PsCKX* 930 (*PsCKX4*) and *PsCKX* 942 (*PsCKX5*).

The phylogeny was done by aligning nucleotide sequences of some of the legumes and *A.thaliana*. The isolated *PsCKX1* grouped with *PsCKX1* and *GmCKX7* and *AtCKX5*. The

isolated *PsCKX5* grouped with *GmCKX5* and *AtCKX6*. The isolated *PsCKX4* and *PsCKX2* formed a clade with *AtCKX1*, *GmCKX1* and *MtCKX*. The isolated *PsCKX3b* grouped with *GmCKX2* and *GmCKX3*. Another putative *PsCKX3* branched with *GmCKX3*, though it was not sequenced. *AtCKX2*, 3 and 4 formed a separate sub clade of the legume CKX3 gene families.

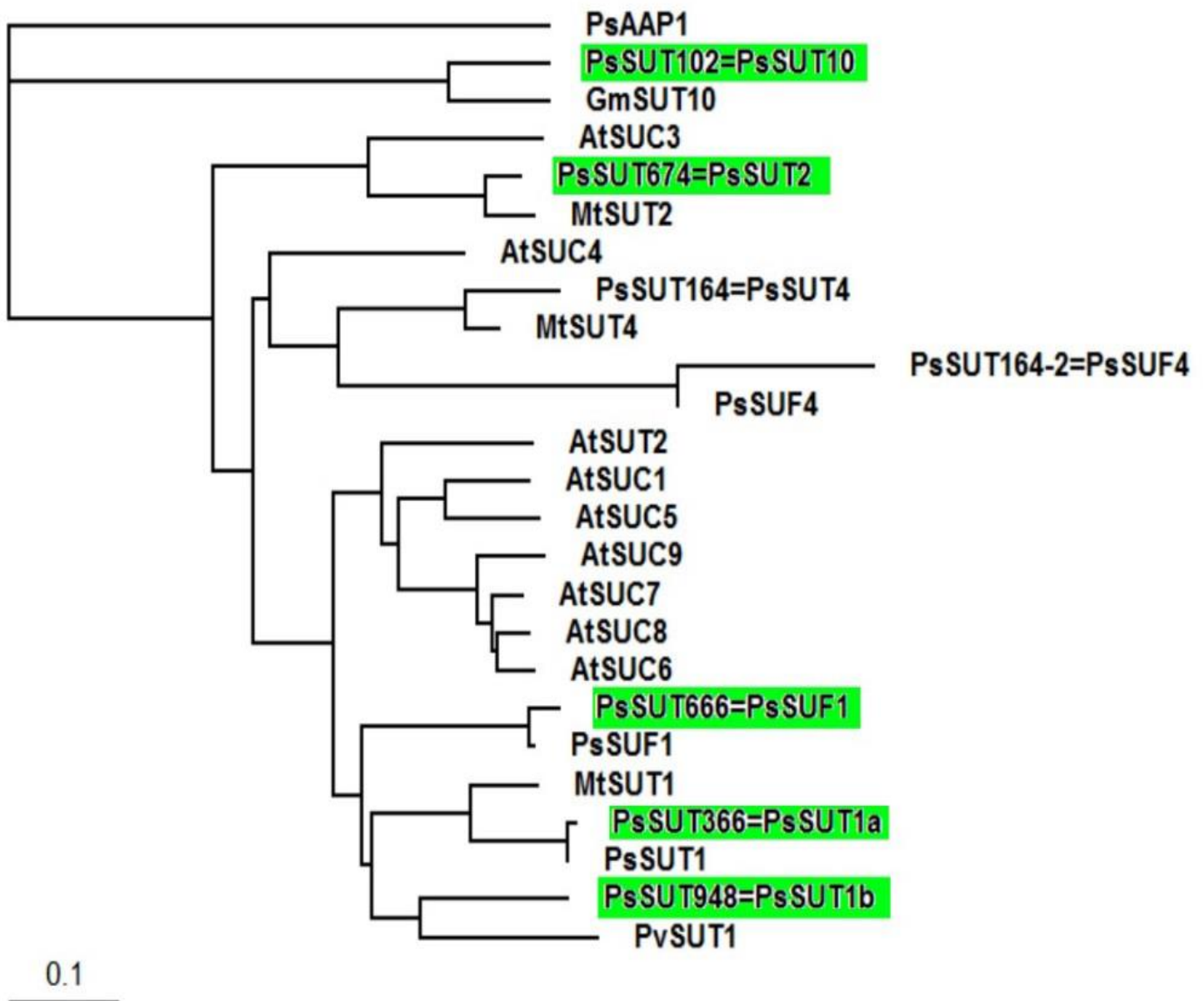


**Figure 3.5: Phylogenetic tree of CKX.** The *PsCKX* gene family members isolated and used in this study are highlighted. The tree was rooted with *RfCKX*.

### 3.3.2.3 Sucrose transporter (SUT) gene family

The phylogenetic analysis was done by including the sequences of *A. thaliana*, *P. sativum* and other legumes including *M. truncatula*, *G. max* and *P. vulgaris* for SUT genes along with the isolated *PsSUT* gene families by MEGA4 program. The tree was rooted with *PsAAP1* as an out group.

When the phylogeny was completed, the tree showed five main clades. The isolated *PsSUT10* grouped with *GmSUT10*. The isolated *PsSUT2* was grouped with *MtSUT2* and *AtSUC3*. *PsSUT4* and *PsSUF4* formed a clade with *AtSUC4* and *MtSUT4*. All *Arabidopsis* SUC gene family members (*AtSUC1*, 5-9) and *AtSUT2* grouped into a separate clade. The isolated *PsSUF1*, *PsSUT1a* and *PsSUT1b* were grouped together with *MtSUT1* and *PvSUT1*.



**Figure 3.6: Phylogenetic tree of SUT.** The *PsSUT* gene family members isolated and used in this study are highlighted. The tree was rooted with *PsAAP1*.

### ***3.3.2.4 Amino acid permease (AAP) gene family***

The phylogenetic tree of *PsAAP* was rooted with *PsSUT1* as an out group. The phylogeny showed that the fifteen *PsAAP* genes isolated had sequence similarity with legume AAPs and *A. thaliana* AAPs. *AtAAPs* mainly branched into 4 clades. The isolated *PsAAPs* were dispersed across all the clades. *AtAAP1*, 6 and 8 grouped together in a clade where *CaAAP6*, *GmAAP6* and the isolated *PsAAP6c* and *6d* were present.

The phylogenetic analysis revealed that the isolated *PsAAP3a* had sequences homologous to *PsAAP1*, whereas, the isolated *PsAAP1* had high sequence similarity with *CaAAP1*. The isolated *PsAAP3a* and *3b* grouped to a clade with *PsAAP1*, *GmAAP3*, *CaAAP3*, *AtAAP3* and *AtAAP5*. The isolated *PsAAP2a*, *2b*, *2c*, *2d* and *2e* formed a clade with *PsAAP2*, *CaAAP2* and *GmAAP2*. The isolated *PsAAP7b*, *7c*, *7d* and *7e* grouped with *AtAAP7*, *CaAAP7* and *GmAAP7* in a clade.

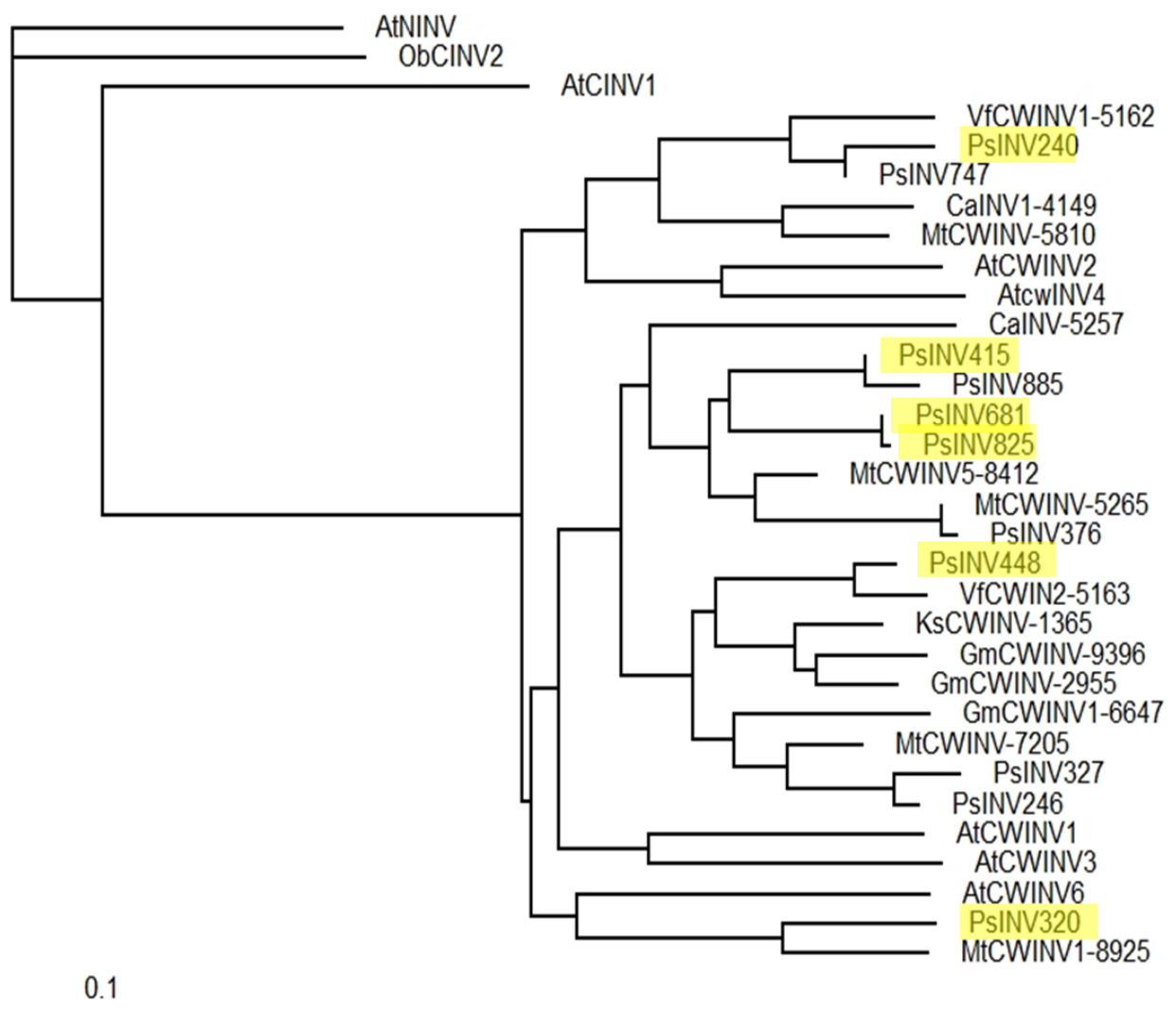




### 3.3.2.5 Cell Wall Invertase (CWINV) gene family

Six *PscwINV* gene family members were isolated and identified. The phylogenetic analysis was done by selecting the nucleotide sequences of *cwINV* genes of *A. thaliana*, *P. sativum* and other legumes including *V. faba*, *M. truncatula*, *G. max*, along with isolated *PscwINV* gene families by the MEGA4 program. The tree was rooted with neutral and cytoplasmic invertase of *A. thaliana* (*AtNINV*, *AtCINV1*).

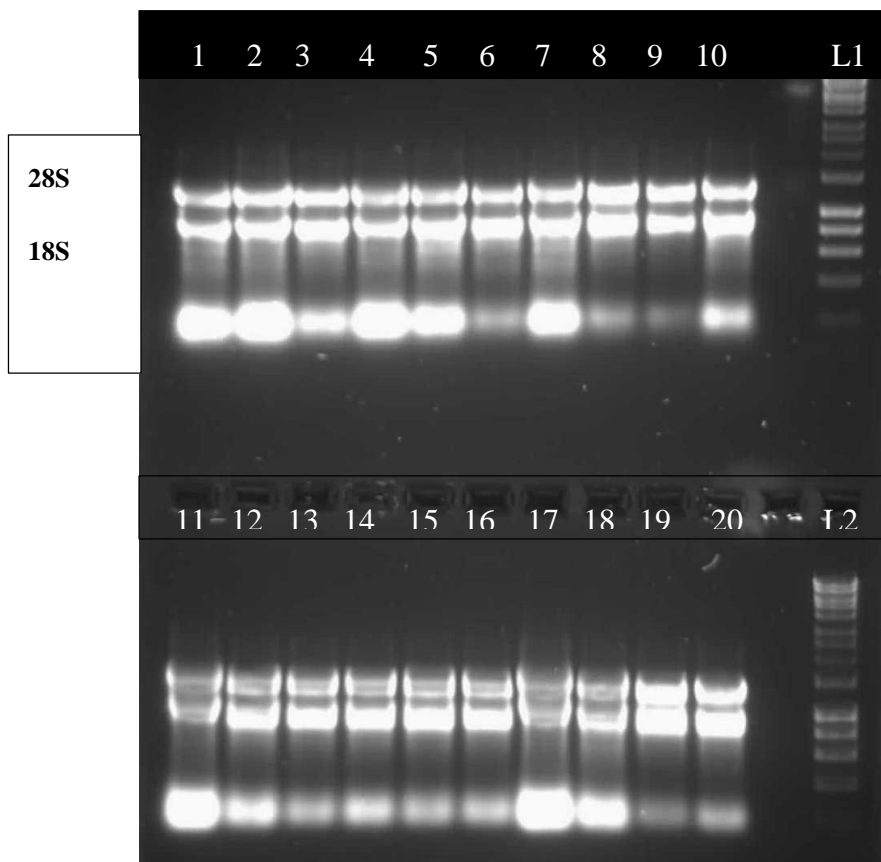
When the phylogeny was completed, the tree branched into five main clades.



**Figure 3.8: Phylogenetic tree of *cwINV*.** The *PsINV* gene family members isolated and used in this study are highlighted. The tree was rooted with *AtCINV1*.

### 3.3.4 Optimisation of RT-qPCR

Total RNA extracted from all the tissue samples by using the TRIzol method normally gave very good yield (0.9-3.5 ng/ $\mu$ l) and good quality RNA with 260/280 ratio of 1.8-2.1 in TE buffer. The integrity of extracted RNA was assessed by using gel electrophoresis in which good quality RNA showed two bands of 18S and 28S ribosomal RNA (Figure 3.10). cDNA was only synthesized from the good quality RNA.



**Figure 3.9:** Total RNAs extracted using TRIzol reagent. 1-10 are the cotyledon samples and 11-20 are pod wall samples. L1 and L2 are DNA ladders.

#### 3.3.4.1 Optimisation of Reference genes

To calculate the target gene expression in RT-qPCR, two or three reference/ housekeeping genes were used. The reference genes used in this experiment were *PsEF* (Elongation Factor), *PsGAP* (glyceraldehyde-3-phosphate dehydrogenase) and *PsACT* (Actin). The

degenerate primers for these reference genes were designed and sequenced by another member of our lab group. The primer sequences for these genes were:

### ***PsEF***

PsEF F2: 5' AGAATGTTGCAGTCAAGGATCTCAAG

PsEF R2: 5' CTCCTTCTCAAKCTCCTTACCAGATC

### ***PsGAP***

PsGAP F: 5' GGTATGTCATTCCGTGTCCCA

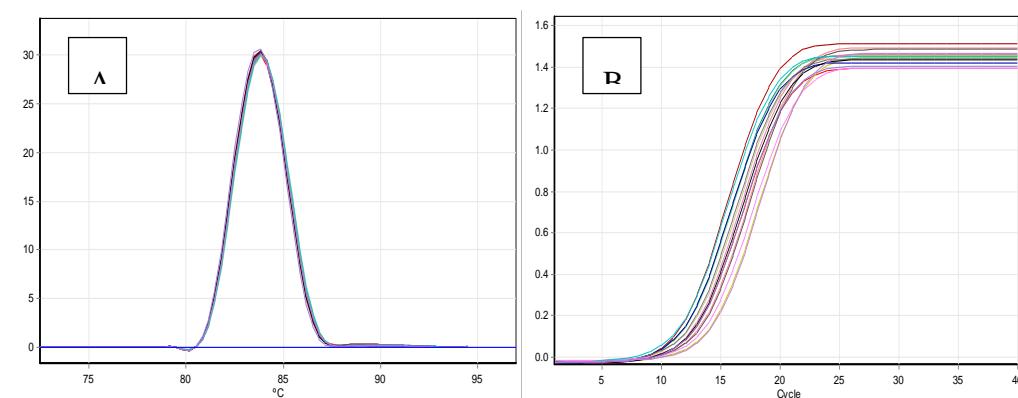
PsGAP R: 5' CCCTCAGACTCTTCCTTGATAGC

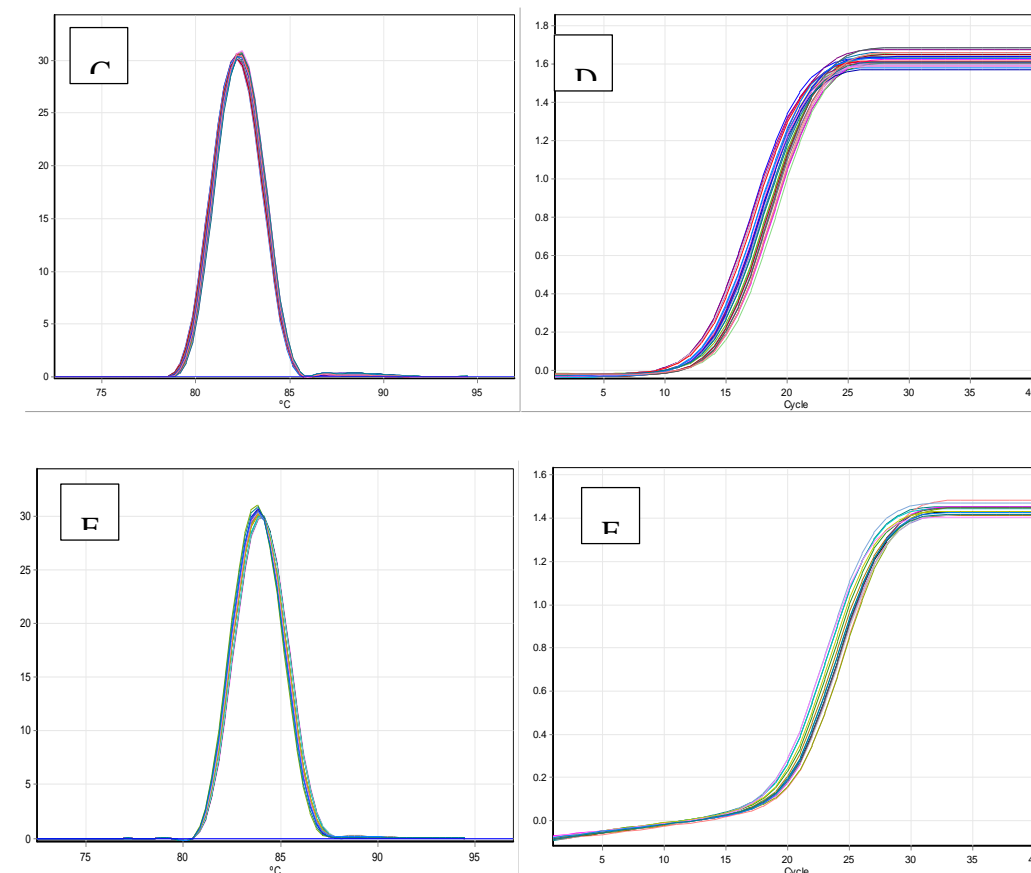
### ***PsACT***

PsACT F1: 5' TTGGATTCTGGTGATGGTGTG

PsACT R1: 5' CATAGATGGCTGGAAAAGGAC

Using cDNAs of different stages of cotyledons, the melt curve of all the reference genes produced a single sharp peak and the Ct values were within 1-3 cycles [Figure 3.11].





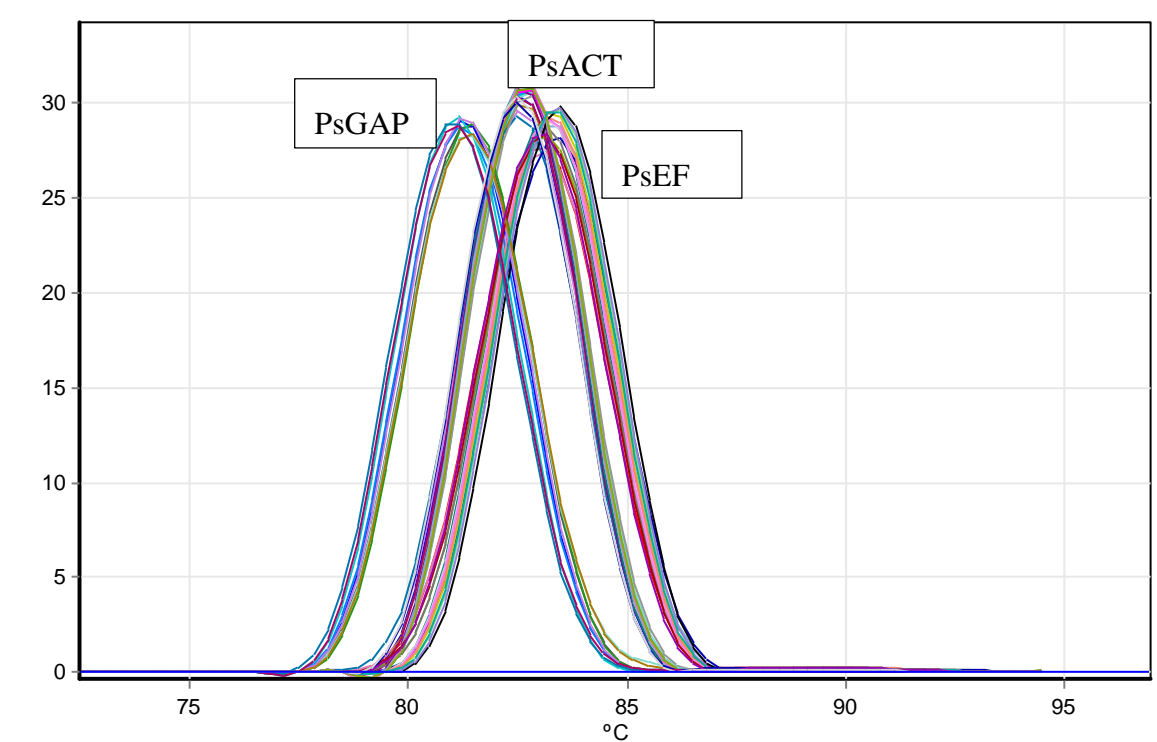
**Figure 3.10: RT-qPCR profiles of reference genes.** Melt curve and amplification curve of *PsEF* (A-B), melt curve and amplification curve of *PsACT* (C-D), melt curve and amplification curve of *PsGAP* (E-F). cDNAs used for these reactions were early stages of developing seeds

### 3.3.4.2 Normalisation of cDNAs

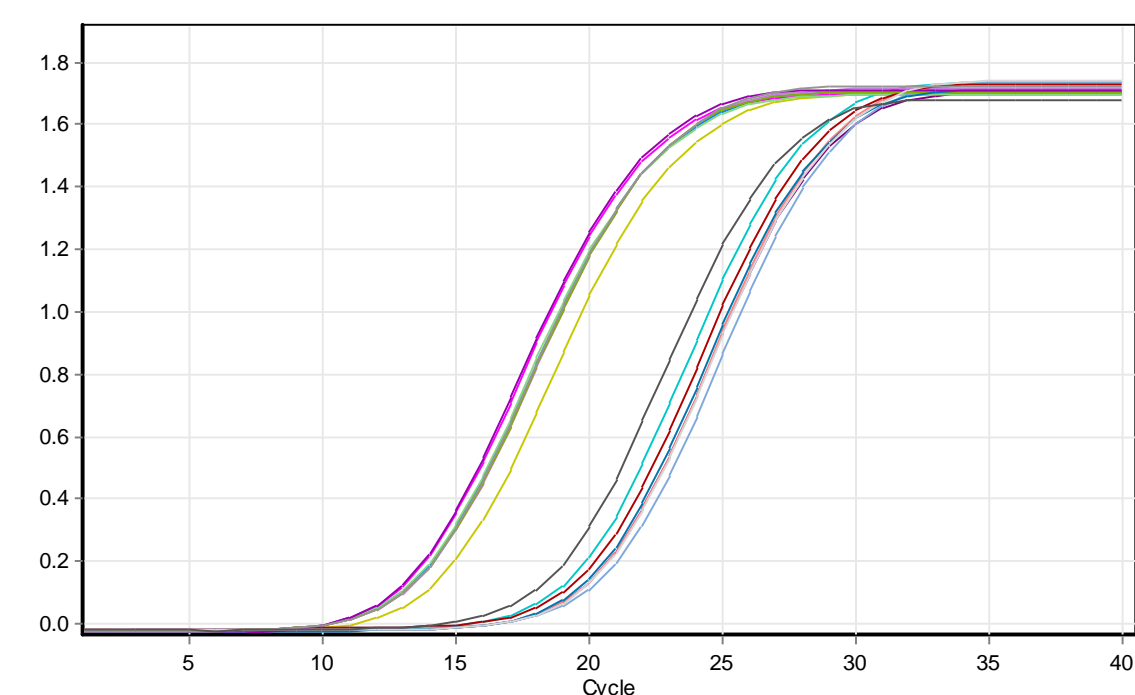
The quality of cDNA synthesized was checked by doing RT-qPCR. The three reference genes used to check the quality and concentration of each cDNA were *PsEF*, *PsGAP* and *PsACT*.

Before expression studies for the target genes were done, each set of cDNAs was normalised using the reference genes, with three technical replicates. Normalisation was done in such a way that the Ct (threshold cycle) values between each cDNA were within a maximum difference of 1-2 cycles. cDNAs were either diluted or resynthesized to ensure Ct values were within the acceptable range. Ct values for *PsEF* and *PsACT* were generally between 10-15, whereas for *PsGAP* they were between 15-18.

Optimisation of primers and cDNAs was performed by determining the annealing temperature of all target primers and reference genes at 52°C, 55°C, 58°C and 60°C. Among all these temperatures, 55°C and 58°C were the best for most of the target primers [Figures 3.12 and 3.13].



**Figure 3.11: RT-qPCR melting curves for reference genes PsEF, PsGAP and PsACT at 58°C.**



**Figure 3.12: RT-qPCR amplification curves for reference genes PsEF, PsGAP and PsACT at 58°C.**

### 3.3.4 Gene expression analysis

All the melting and amplification curves of different gene family members (GFM) are shown in the Appendix, Section 3.1.

#### 3.3.4.1 Expression analysis of *PsIPT* gene family members

The relative expression of cytokinin biosynthesis and degradation genes was studied in both process pea and field pea during different stages of seed development. In this experiment, the developmental stages were grouped into two; they were the morphogenesis (early) stages (-1 to 10 DAF) and maturation (later) stages (12 to 30 DAF) of seed development.

Out of the three isolated *PsIPT* gene family members (GFM), *PsIPT2* and *PsIPT4* showed differential expression patterns with time and tissue (Figure 3.13 A-D). *PsIPT2* showed

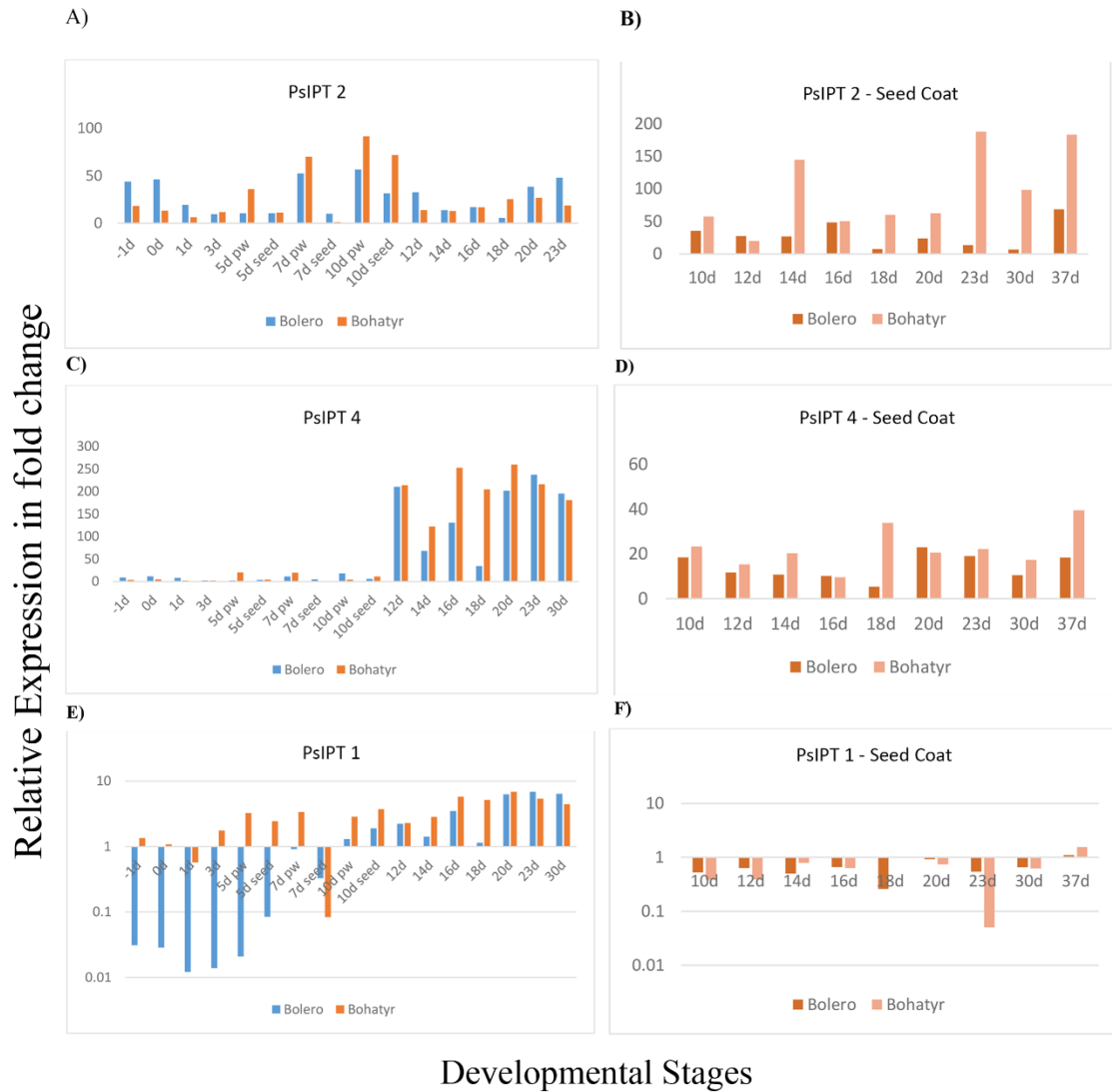
greater expression during morphogenesis phase, whereas, *PsIPT4* was up-regulated during the maturation stages of cotyledon development.

In process peas, *PsIPT2* was more highly expressed at the very early stages (-1, 0 DAF) and in pod walls of 7 and 10 DAF. This gene family member subsequently showed elevated expression in pod walls of field pea. The expression level then decreased and was again up-regulated during the final stages of cotyledon development (20, 23 DAF). Interestingly, process peas showed higher expression than field peas at this later stage. Additionally, gene expression was analysed in seed coats of both the cultivars from 10 to 37 DAF. *PsIPT2* was more up-regulated in the seed coats of field pea, than in process pea, especially, at the later stages (Figure 3.13 B).

*PsIPT4* showed more expression during the later stages of seed development (12- 30 DAF) (Figure 3.13 C). From 14 to 20 DAF, field pea had elevated expression compared to process pea which had slightly higher expression at 23 and 30 DAF. At the same time, in seed coats, this gene family member was expressed at 10 DAF in Bolero, and then the expression decreased till 18 DAF. At the later stages, both process pea and field pea showed elevated expression. But generally, field pea showed higher expression than process pea.

*PsIPT1* showed extremely low expression throughout development, when compared with the other two gene family members.





**Figure 3.13: Relative expression of PsIPT1, 2 and 4 gene family members in developing seeds.** A,B) *PsIPT2* in cotyledons and seed coats, C,D) *PsIPT4* in cotyledons and seed coats, and E,F) *PsIPT1* in cotyledons and seed coats. Data are means of relative mRNA levels in fold changes detected using three technical replicates. X-Axis is the developmental stages starts from -1 to 30 DAF, in which -1 to 3 DAF is the whole pod with seeds in it, 5 to 10 DAF gives separated seeds from the pod wall, 12 to 30 DAF is pure cotyledons without seed coat.

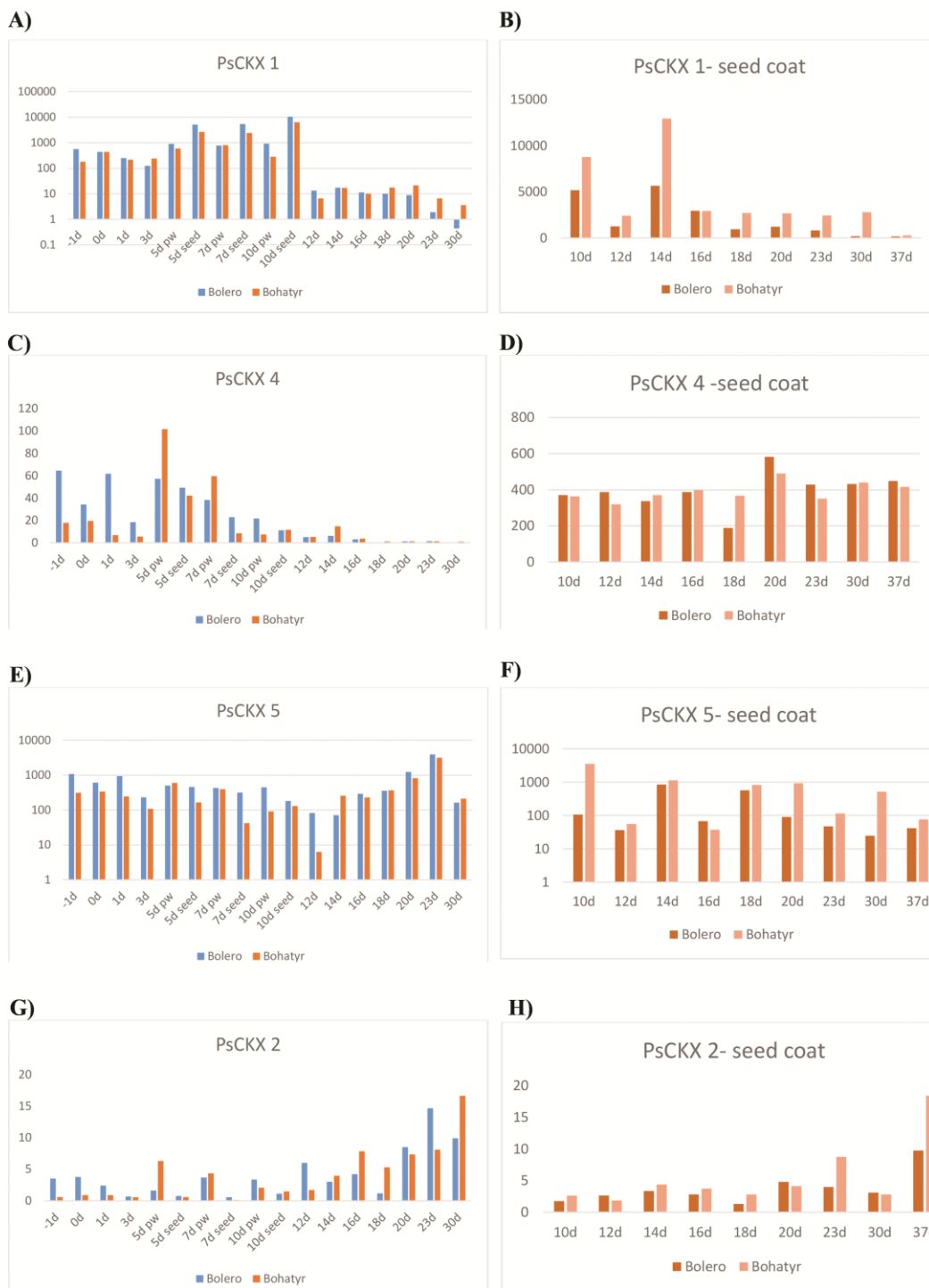
#### ***3.3.4.2 Expression analysis of PsCKX gene family members***

Among the five isolated gene family members, only three showed differential expression patterns with time and tissue (Figure 3.14A-F). *PsCKX1* was expressed strongly in the young seeds (5, 7 and 10 DAF) (Figure 3.15A, note log scale). Process pea showed greater expression than field pea. In contrast, in seed coats, this gene family member was highly expressed in the seed coats of field pea, especially at 10 and 14 DAF compared with process pea.

*PsCKX4* showed elevated expression pattern in process peas at the very early stages (-1, 0, 1 DAF) (Figure 3.14C). Then the expression decreased and again it was up-regulated especially in the pod walls of field pea. However, by 10 DAF, expression had decreased to low levels in both pod walls and cotyledons (Figure 3.14C). During later stages of development, this GFM was highly expressed in the seed coats of both process pea and field pea, relative to seeds, but much less expressed compared with CKX1.

Another gene family member, *PsCKX5* showed expression in all tissues throughout development (Figure 3.14E, note log scale) with generally greater expression in process peas. At the final stage of seed development (20 and 23 DAF) this gene is up-regulated in Bolero. In the case of seed coats, this gene is highly expressed in Bohatyr, especially at 10 DAF after which the expression fluctuated. However, field peas generally showed greater expression of this gene in seed coats, than process pea.

*PsCKX2* showed very low expression in both the cultivars at all stages (Figure 3.14G, H).



**Figure 3.14: Relative expression of PsCKX1, 2, 4, and 5 gene family members in developing seeds.** A,B) *PsCKX1* in cotyledons and seed coats, C,D) *PsCKX4* in cotyledons and seed coats, E,F) *PsCKX5* in cotyledons and seed coats, G,H) *PsCKX2* in cotyledons and seed coats. Data are means of relative mRNA levels in fold changes detected using three technical replicates.

#### ***3.3.4.3 Expression analysis of sucrose transporter gene family members***

Five *PsSUT* gene family members were identified and isolated in this study. Among them, one is most likely a sucrose facilitator (*PsSUF1*). Both process pea and field pea have shown almost similar patterns of expression for all the *PsSUT* family members (Figure 3.15).

*PsSUF1* showed higher expression predominantly in young seeds of both process pea and field pea, with process pea generally showing higher expression than field pea, and seeds show elevated expression compared with pod walls. When expression of this gene family member was studied in seed coats of both process pea and field pea, elevated expression was shown in both the cultivars only at 10 and 12 DAF, with process pea showed higher expression than field pea at 10 DAF.

*PsSUT1a* was very strongly expressed during the early stages of seed development. Interestingly, strong expression was seen in the pod walls of both process pea and field pea. Expression reduced in the cotyledons at 14 and 16 DAF but increased again in the later stages. In the case of seed coats, the gene family member was highly expressed in both process pea and field pea at all stages with elevated expression in mature seed coats at 23 and 30 DAF.

*PsSUT1b* showed a similar pattern of gene expression to *PsSUT1a* in the young seed, pod walls and cotyledons. However, in the seed coats, the expression pattern of *PsSUT1b* differed from that of *PsSUT1a*. The expression remained steady until 18 DAF in both the cultivars, and then expression increased to a maximum at 30 and 37 DAF. Process pea showed higher expression than field pea at these latter times.

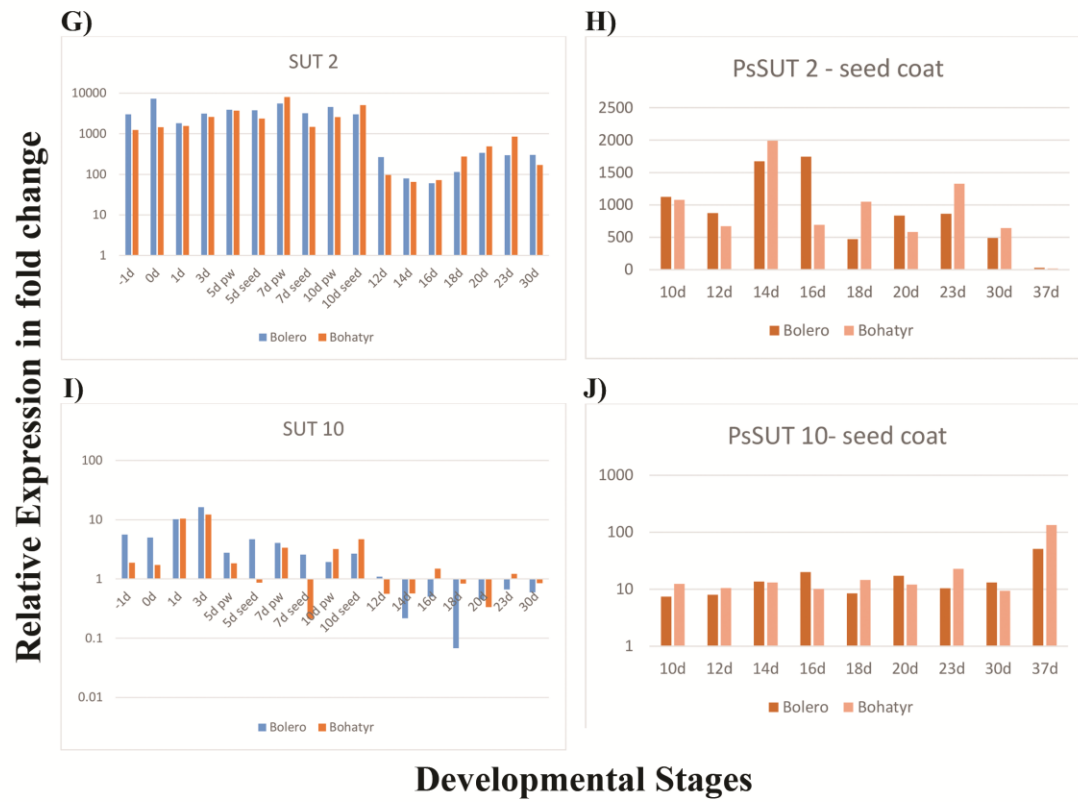
Elevated expression of *PsSUT2* was seen in the very young pods (-1, 0 DAF) of process pea. And again, reduced expression was shown in cotyledons of both the cultivars. When gene expression was studied in seed coats, the highest expression was shown in process pea at 14

and 16 DAF and field pea at 14 DAF. This gene family member showed greater expression in process pea compared with field pea at 10, 12, 16 and 20 DAF whereas the field pea showed greater expression at 14 and 18 DAF as well as in the mature seed coats.

In comparison with the other four gene family members, *PsSUT10* exhibited very low expression in early and later stages of seed development. This GFM was expressed in seed coats, at a relatively low level compared to other SUT gene family members.

Relative Expression in fold change





**Figure 3.15: Relative expression of PsSUT1a, 1b, 2, 10 and PsSUF1 gene family members in developing seeds and seed coats. a,b) *PsSUF1* in cotyledons and seed coats, c,d) *PsSUT1a* in cotyledons and seed coats, e,f) *PsSUT1b* in cotyledons and seed coats,**

*g,h)PsSUT2* in cotyledons and seed coats, *i,j) PsSUT10* in cotyledons and seed coats. Data are means of relative mRNA levels in fold changes detected using three technical replicates.

#### ***3.3.4.4 Expression analysis of amino acid permease gene family members***

Fifteen *PsAAP* gene family members were identified and isolated. Of these, fourteen gene family members showed expression during seed development, while one member (*PsAAP8*) showed little expression throughout seed development (Figure 3.16).

During early development *PsAAP1* showed greater expression in pod walls compared to seeds, but expression in the cotyledons increased between 10 and 16 DAF and then stabilised. The data shows strong expression in the seed coats between 10 to 14 DAF with this GFM generally more highly expressed in field pea than process pea. Greater expression of this GFM was seen in Bohatyr at 10, 12 and 14 DAF, then the expression decreased and again during final stages, an elevated expression was shown by field pea.

Three of the *PsAAP2* family members (2a, 2b and 2d) showed similar expression patterns, that is, higher expression during early stages of seed development than the later stages. Similarly, these gene family members exhibited slightly elevated expression in pod walls compared with seeds. *PsAAP2e* showed different pattern of expression. This member was strongly expressed in pod walls relative to other tissues and stages of development.

The greatest expression in seed coats was shown by *PsAAP2b* whereas, *PsAAP2e* showed hardly any expression. Expression of *PsAAP2a*, 2b and 2d was more elevated in seed coats of field pea compared to process pea at 23 and 30 DAF.

*PsAAP3b* expressed at a considerably greater level than *PsAAP3a* in the early stages of seed development. *PsAAP3b* showed hardly any expression in cotyledons during 12, 14, 16 and 18 DAF, whereas *PsAAP3a* showed low but increasing expression at this stage. The data also reveals that, in seed coats, both the gene family members showed similar trend. Expression of both gene family members increased from 23 DAF in field peas and process peas.

*PsAAP6c* showed higher expression in both process pea and field pea in early developing seeds (5, 7 and 10 DAF) unlike *PsAAP6d* and other gene family members, where expression

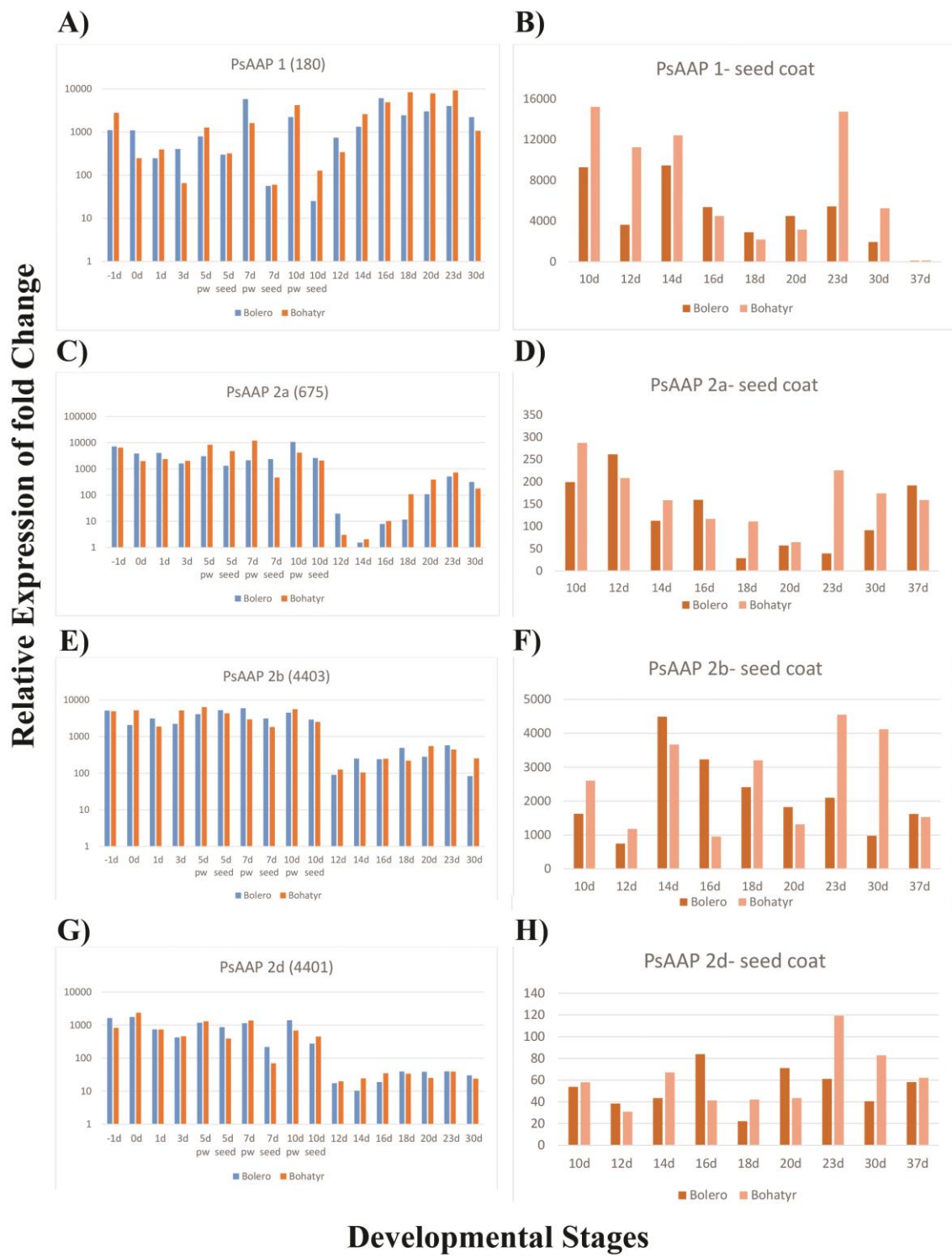


was greater in the pod walls. In the case of seed coats, both *PsAAP6c* and *6d* were expressed at the same level in Bolero and Bohatyr at 10 and 12 DAF. Then expression increased in Bolero at 16, 20 and 23 DAF. At 23 and 30 DAF, both the gene family members were expressed more highly in seed coats of Bohatyr compared with Bolero.

Among the gene family *PsAAP7*, *7b* and *7e* showed very similar expression pattern, expressing strongly in pod walls and with little expression in the cotyledons. During the early and later stages, *7c* showed strong expression, whereas *7d* was only weakly expressed. Interestingly, in seed coats, each member expressed differently. *PsAAP7b* showed elevated expression during 10, 12 and 14 DAF for field pea and then the expression decreased. This gene family member showed hardly any expression in mature seed coats. In contrast, *7c* was expressed only in field pea at 10 and 12 DAF. Then the expression was low until at 37 DAF, both Bolero and Bohatyr showed expression of this gene. *PsAAP7d* had very low expression at 10 and 12 DAF, then it increased to 20 DAF. At 23 DAF, expression of this gene was higher in Bohatyr. Finally, low expression was shown at 37 DAF. Another noticeable finding was that *PsAAP7d* was hardly expressed in the cotyledons from 12-30 DAF, whereas this gene showed much stronger expression in seed coats at the same stages. *PsAAP7e* showed a steady expression from 10 to 16 DAF and from 18d it increased in seed coats. Field pea showed an elevated expression at 23 and 30 DAF. Expression of both gene family members had reduced by 37 DAF.

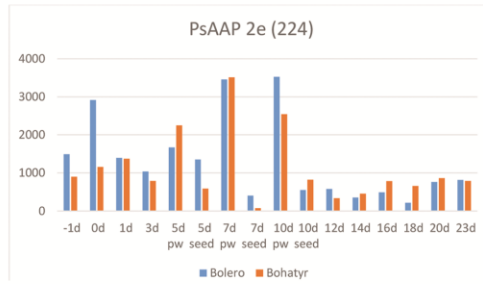
*PsAAP8* showed extremely low expression throughout the seed development.

A)

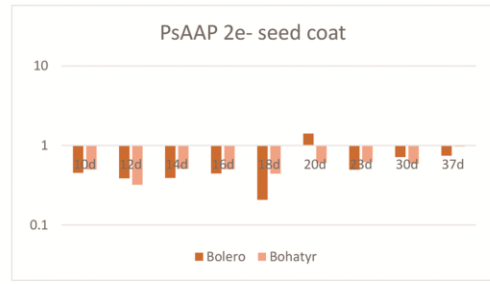


Relative Expression in fold change

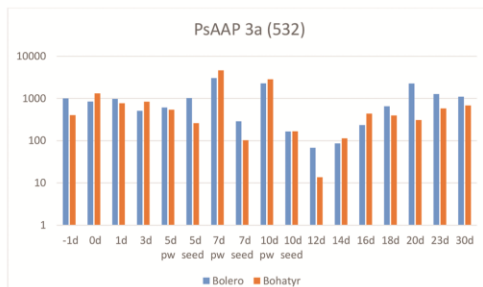
I)



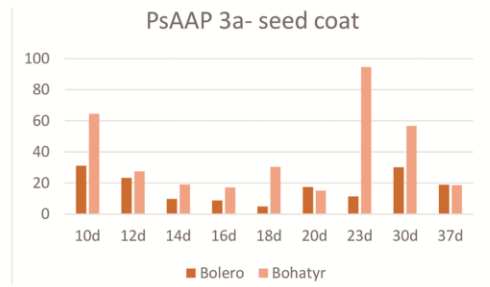
J)



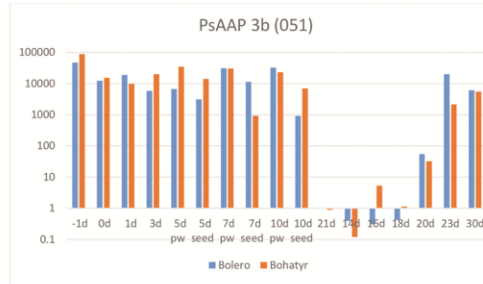
K)



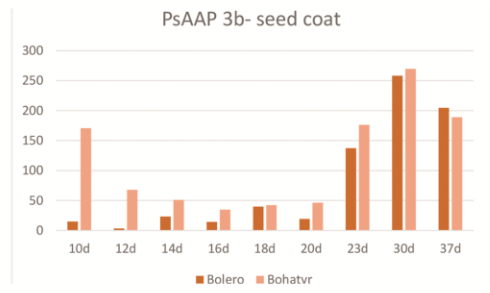
L)



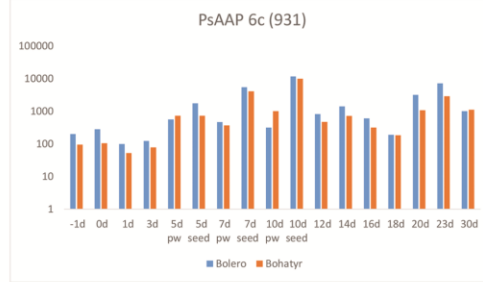
M)



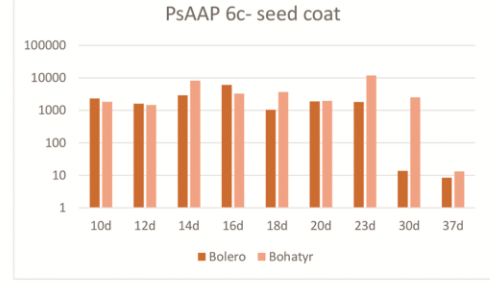
N)



O)

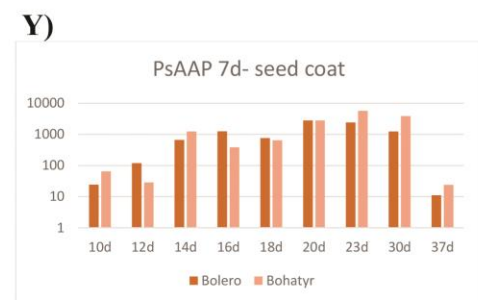
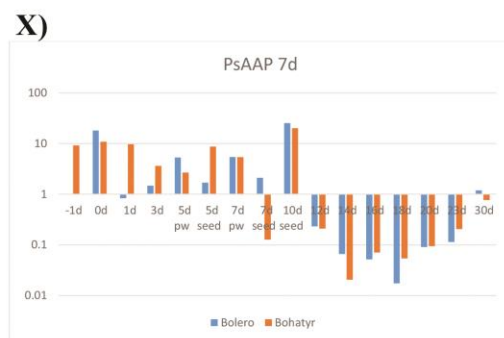
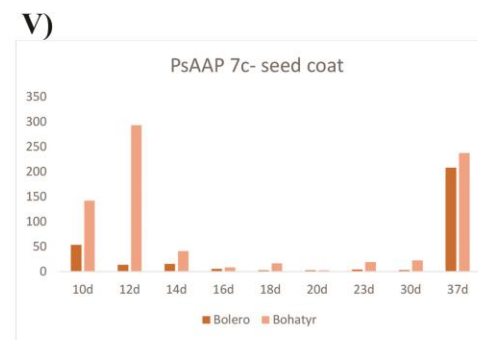
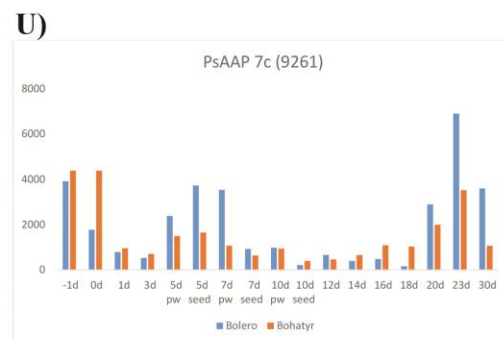
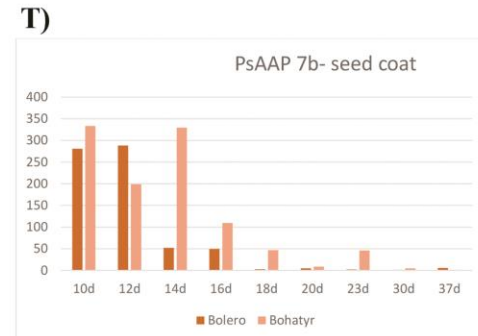
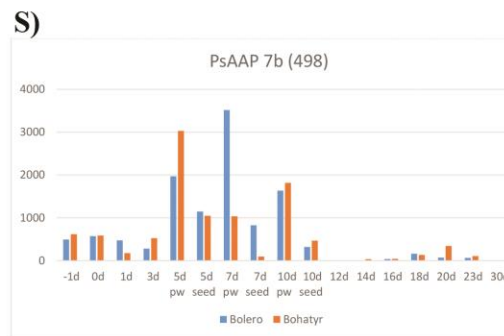
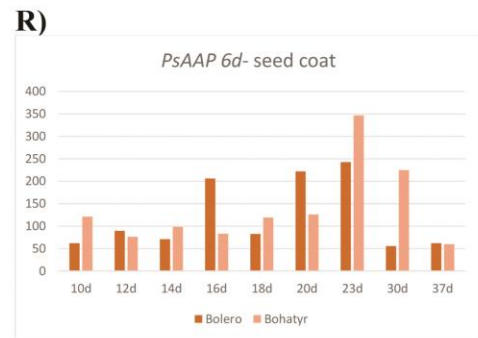
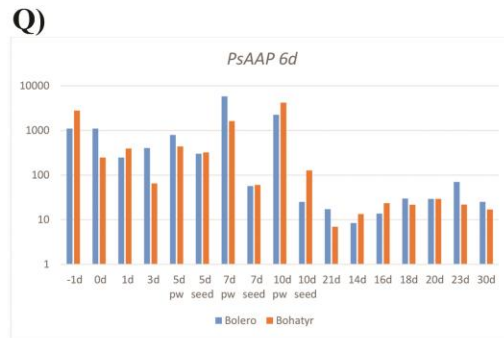


P)

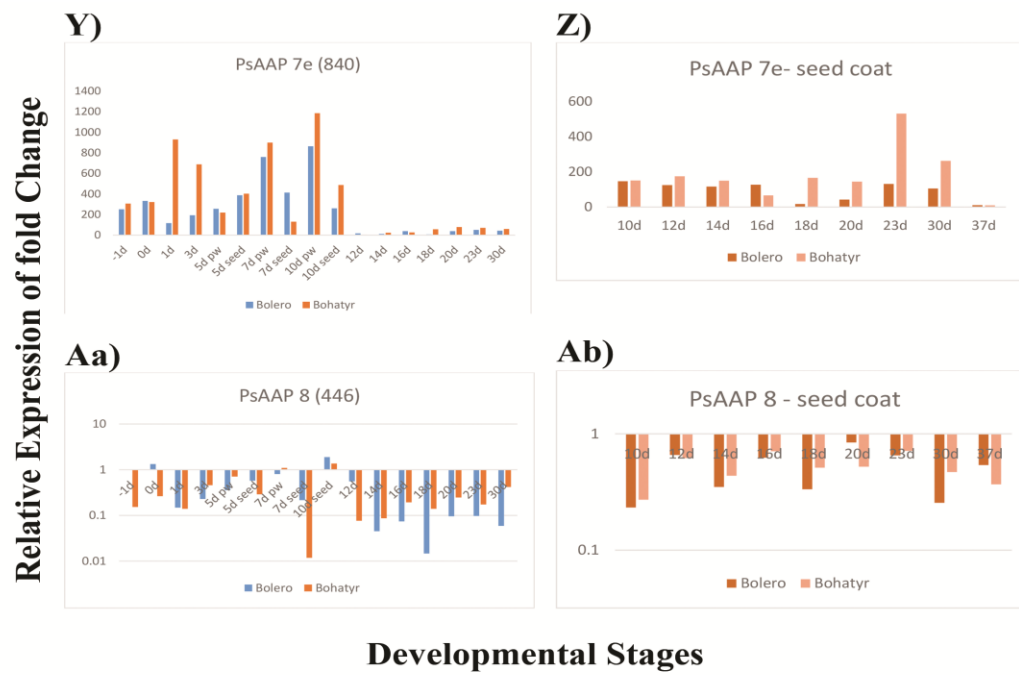


Developmental Stages

Relative Expression in fold change



Developmental Stages



**Figure 3.16: Relative expression of PsAAP gene family members in developing seeds and seed coats.** *a,b)* *PsAAP1* in cotyledons and seed coats, *c,d)* *PsAAP2a* in cotyledons and seed coats, *e,f)* *PsAAP2b* in cotyledons and seed coats, *g,h)* *PsAAP2d* in cotyledons and seed coats, *i,j)* *PsAAP2e* in cotyledons and seed coats, *k,l)* *PsAAP3a* in cotyledons and seed coats, *m,n)* *PsAAP3b* in cotyledons and seed coats, *o,p)* *PsAAP6c* in cotyledons and seed coats, *q,r)* *PsAAP6d* in cotyledons and seed coats, *s,t)* *PsAAP7b* in cotyledons and seed

coats, U,V) *PsAAP7c* in cotyledons and seed coats, W,X) *PsAAP7d* in cotyledons and seed coats, Y,Z) *PsAAP7e* in cotyledons and seed coats, Aa,Ab) *PsAAP8* in cotyledons and seed coats. Data are means of relative mRNA levels in fold changes detected using three technical replicates.

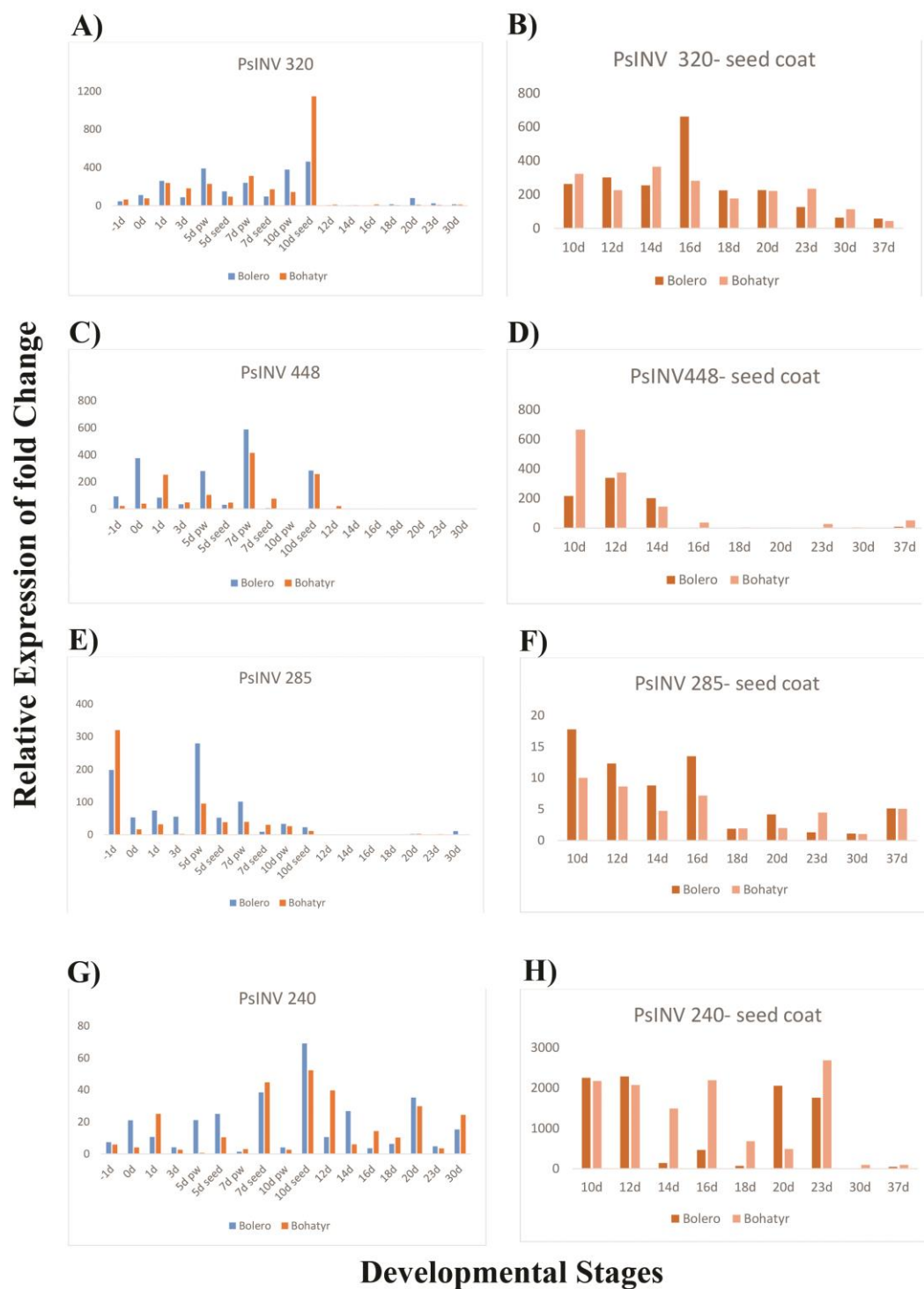
#### ***3.3.4.5 Expression analysis of cell wall Invertase gene family members***

Three of the isolated *PscwINV* gene family members (PsINV320, PsINV448 and PsINV285) exhibited greater expression during early stages of seed development but reduced expression during the maturation phase. In addition, PsINV320 showed elevated expression in the developing seeds (10 DAF) of Bohatyr, whereas PsINV448 was more highly expressed in the very young pods (-1, 0 DAF) and the pod walls of Bolero. Unlike these three gene family members, PsINV240 showed continuous expression throughout the development with particularly high expression in young seeds (7, 10 DAF) of both process pea and field pea, compared to pod walls.

In seed coats, all the *PsINV* gene family members expressed at 10, 12, and 14 DAF. From 16 DAF, expression of PsINV320 decreased slowly. PsINV448 showed high expression at 10 DAF in Bohatyr. Then it decreased and from 16 DAF, expression was low. Comparatively, field pea had higher expression than process pea at 10 DAF. PsINV285 showed higher expression in Bolero than Bohatyr from 10- 16 DAF. Then gene expression decreased in both the cultivars. And finally at 37 DAF, Bolero and Bohatyr showed similar level of expression.

Among invertase genes, PsINV240 was the most highly expressed gene family member in seed coats. This gene was strongly expressed in both Bolero and Bohatyr at 10-12 DAF. Then the expression decreased in process pea, whereas, the expression was at the same level in Bohatyr till 18 DAF. At 20 and 23 DAF, expression in Bolero increased. In mature seed coats (30 and 37 DAF), expression of this gene family member was very low.

PsINV415 and PsINV681 did not show expression at any stage in developing seeds.



**Figure 3.17: Relative expression of PsINV gene family members in developing seeds and seed coats. a,b) *PsINV320* in cotyledons and seed coats, c,d) *PsINV448* in cotyledons and seed coats, e,f) *PsINV285* in cotyledons and seed coats, g,h) *PsINV240* in cotyledons and**

seed coats. Data are means of relative mRNA levels in fold changes detected using three technical replicates.

## **3.4 Discussion**

In this chapter, expression of genes affecting assimilation and yield was assessed in the two different genotypes of pea, process pea (Bolero) and field pea (Bohatyr).

### **3.4.1 Early and later stages of seed development in process and field pea**

A full phenotypic analysis was carried by Dr Jan Grant. Visual analysis indicated that there were some differences during growth. Bolero, the process pea plants were bushy and had wide leaves. Bohatyr plants were taller than Bolero and their leaves were smaller. Flowering started earlier in Bolero, than Bohatyr, the field pea.

In this experiment, seed samples of different stages were analysed, throughout development. Studies have shown that there are two important phases of legume seed growth (Wang, 1993). The first growth phase is limited to endosperm and seed coat, while the second phase is associated with embryo divisions. The first phase is characterized by cell division and differentiation, also called morphogenesis stage and the second stage is maturation phase which is characterized by expansion and accumulation of storage products.

In this study, samples collected of early developmental stages included pods and/or seeds from -1, 0, 1, 3, 5, 7 and 10 DAF which means that these samples were collected during the morphogenesis phase of development. The cotyledons studied in this experiment were collected from 12- 30 DAF, which was during the maturation stage. Hence, a clear picture of expression of different genes during seed development was obtained. The cotyledon samples analysed were collected by dissecting the pods and individual seeds. While dissecting, it was noticed that the seed contained liquid endosperm till 10 DAF, so the water content was



around 90%. As the seed matured, the growing embryo and cotyledon were nourished from the endosperm which gradually disappeared.

Results from the starch/ sugar analysis revealed that there were noticeable differences between cotyledon and seed coat profiles. Both in field peas and process peas, the cotyledons contained greater amounts of sucrose than seed coats during early stages. Then at the maturation stage, sucrose was much reduced or below the limit of detection in both tissues. In the case of starch, during early stages, the cotyledons of field pea contained higher amounts of starch than did those of process pea. As seed matured, the amount of starch increased in field pea and was higher at the latest stages. Process pea contained negligible amount of starch in the beginning and gradually increased. During maturation phase, some of starch was present in process pea cotyledon but less than field pea, whereas seed coats of both cultivars showed a small amount of starch during early stages then no starch was present in the matured seed coats.

### **3.4.2 Identification, characterization and expression of *IPT* gene**

In this experiment, gene family members of coding for the key cytokinin biosynthetic enzyme, IPT, was isolated from *P. sativum*. The relative expression of the various gene family members was studied to understand the spatial and temporal expression pattern of these genes. The multi-gene family members were initially isolated following the analysis by Dr Jason Song of a pea transcriptome. Some genes that were detected in the pea transcriptome were not selected for the final expression studies since their expression was very low or undetectable following RT-qPCR analysis. But it is important to note that if a gene family member is undetectable by RT-qPCR method, it does not mean that its product is non-functional within the plant system as it may only be required at very low levels to induce a response. However, an assumption was made that those undetected gene family members probably do not play any crucial role in the developmental stages and tissues studied in this experiment.

The phylogenetic analysis of the *IPT* gene family members showed that the dicot genes had two distinct clades, one containing *AtIPT5*, *AtIPT7* along with legumes *PsIPT1*, *PsIPT2*, *LjIPT4* and *GmIPT5* and the second clade containing *AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT6*, *AtIPT8* along with legumes *PsIPT3*, *CaIPT1*, *CaIPT3*, *GmIPT3*, *GmIPT5*, *LjIPT3*. Hence,

from this result it is evident that the isolated *PsIPT1*, *PsIPT2* and *PsIPT3* are homologous with *AtIPTs* and other legume *IPTs*.

In *Arabidopsis*, the *IPT* genes are expressed in several tissues, including roots, leaves, stems, flowers and siliques (Miyawaki et al., 2004). Evidence revealed that *AtIPT3* is expressed in phloem tissue in rosette leaves. *AtIPT5* was expressed in lateral roots, upper parts of young inflorescence and fruit abscission zones (Takei et al., 2004, Sakakibara et al., 2006). In addition, it was also shown that two *AtIPT* genes, *AtIPT4* and *AtIPT8* were localized to the endosperm, exclusively in chalazal region up to the early heart stage in *Arabidopsis* (Belmonte et al., 2013) (Miyawaki et al., 2004). In maize, it was shown that the endosperm is a site of *ZmIPT2*, suggesting its role in establishing sink strength and seed development (Brugière et al., 2008).

In this experiment, the results showed that the isolated *PsIPT4* was expressed during the later stages of seed development, which can be related to a result from Emery et al. (2000), which stated that dihydro cytokinin forms were detected not only during early stages of seed development, but a significant level of cytokinin was found throughout seed development. Evidence also showed that total endogenous cytokinin increased during early pea seed development and decreased at maturity (Quesnelle and Emery, 2007a). This trend was consistent with cytokinin accumulation in developing seeds of other legumes such as lupin (Emery et al., 2000), chickpea (Emery et al., 1998), *V. faba* and *G. max* (Quesnelle et al., 2004) and several monocots (Morris, 1997). Expression of isolated *PsIPT2* during early stages in this experiment aligns with to these findings.

The relative expression differs in various tissue types and at different developmental stages (Weber et al., 1997, Schmülling et al., 2003). That means, the tissue specificity of *PsIPT1*, *PsIPT2* and *PsIPT3* was observed in the organs. Le et al. (2012) mentioned that the transcript levels of *GmIPT* in flowers, pods and seeds were different in each tissue which meant that each of these tissues required different *GmIPT* gene family members for cytokinin biosynthesis and that the combined action of at least five *GmCKX* genes is required for maintaining cytokinin homeostasis in flower tissue (Le et al., 2012). O'Keefe et al. (2011) found that in *Brassica rapa*, there was an interesting pattern in the expression of *IPT* and *CKX* genes, that is as *BrIPT* expression increased and decreased, *BrCKX* showed the similar

pattern(O'Keefe et al., 2011a, Song et al., 2015) and it has also been suggested that this is a causative relationship (Galis et al., 2005).

The results showed that *PsIPT2* is highly expressed in the pod walls of Bohatyr during 5, 7 and 10 DAF whereas, in Bolero this gene is up-regulated during the later stages of seed development (20 and 23 DAF). It reveals the spatial and temporal expression of *PsIPT* families. The expression patterns of *AtIPT* genes exhibit considerable variation within the wide range of plant tissues and organs (Miyawaki et al., 2004). Another study showed that expression of *ZmIPT2* was found to be higher from 5 to 25 days after pollination with maximum expression at 10 DAP (Brugière et al., 2008). It was also found that the strong expression of *ZmIPT2* during early stages of development, which coincides with the time of maximum cell division in the endosperm (Brugière et al., 2008). This expression pattern was similar to *AtIPT4* and *AtIPT8* expression during early stages of developing Arabidopsis seeds (Miyawaki et al., 2004).

### **3.4.3 Identification, characterization and expression of *CKX* gene**

The CKX gene family codes for a key enzyme regulating the levels of active cytokinin. The CKX genes show relatively high level of sequence similarity and contain several conserved domains. According to Schmülling et al. (2003) and Ashikari et al. (2005a) the CKX genes group into four clades. The phylogenetic analysis classified the monocot *ZmCKX* to one clade and dicot *AtCKX* with legumes to another clade. The *AtCKX2*, *AtCKX3*, and *AtCKX4* grouped into one clade and *AtCKX5* and *AtCKX7* to another clade. The Arabidopsis genome contains seven CKX gene family members (Bilyeu et al., 2001). The five *PsCKX* genes had sequence similarities with *AtCKX* and legume CKX.

Evidence showed that CKX gene family members could be functionally differentiated by their spatial and temporal expression patterns (Werner et al., 2001, Werner et al., 2003). Also, it was found that *OsCKX2* was expressed in leaves, inflorescence meristems and flowers (Ashikari et al., 2005a). Other literature showed that CKX had an important role in seed development which is clear from work in which CKX was either over or under-expressed in

developing seeds (Kopečný et al., 2006, Werner et al., 2003). Similar result is found in this experiment, that most of the isolated *PsCKX* gene family members showed greater expression during seed development. It was also found that there was a relationship in the expression pattern, that is, when IPT expression increases or decreases, CKX expression follows the same pattern.

The expression profiles of five *PsCKXs* studied in this experiment differed at various stages of development and also in tissues. This finding coincides with the results by Werner et al. (2006) which reported the tissue-specific expression of *AtCKX*. It is also clear from the data that expression of *PsCKX1* was at its maximum during early stages of development and interestingly, it is highly expressed in seed, not in pod wall. This result is related to the statement that cytokinins are often found in high concentration at early stages of seed development, when active cell division takes place (Brenner and Cheikh, 1995). Again, the expression is higher in process peas than that of field peas. Whereas, *PsCKX4* showed elevated expression during later stages of development especially at 12, 14 and 16 DAF. Furthermore, the expression is higher in field peas than that of processed peas. But, at the very last stage (30 DAF) the expression increased in process peas.

The expression levels of *GmCKX* genes in flowers, pods and seeds were studied by Le et al. (2012). It was found that high expression of *GmCKX8* was in pods. The relative expression varied in seeds, pod walls and leaves as Werner et al. (2003) reported about differential expression of *AtCKXs*. The *AtCKX* genes were localized to specific domain in the root and shoot (Werner et al., 2006). *PsCKX1* showed high expression in early developing seeds whereas *PsCKX3* did not show any expression in the developing seeds. Also, the expression was slightly higher in field peas than that of process peas.

#### **3.4.4 Identification, characterization and expression of sucrose transporter (*SUT*)**

Sucrose uptake transporter (*PsSUTs*) multi gene family members were isolated using pea transcriptome data. Through the phylogenetic analysis *PsSUT1a* grouped with *VfSUT1* with high bootstrap value of 99. The *PsSUT1* and *VfSUT1* identical amino acids (Weber et al., 1997) did belong to the same clade when their sequences were compared (Tegeder et al., 1999). The isolated *PsSUT1b* revealed sequence similarities with *PvSUF1*. *PsSUF1*, *PsSUT1b*, *MtSUT1*, *GmSUT1*, *VfSUT* and *PsSUT1a* and grouped into a clade with subgroups

agreeing with Zhou et al. (2007). *PsSUT2* had high sequence similarity with *MtSUT2*, *PsSUT10* and *GmSUT10*.

The latest phylogenetic classification done by Reinders et al. (2012) showed the presence of three main groups of SUTs: Type I, II and III. All Type I and II SUTs are localised to the plasma membrane whereas Type III are located in the tonoplast (Sun et al., 2010, Sivitz et al., 2008). The plasma membrane located SUTs are essential for phloem loading and normal pollen function (Gottwald et al., 2000, Sivitz et al., 2008). The phylogenetic tree done with the isolated *PsSUT* gene family members shows that the gene family members studied in this thesis are members of Type I and II, which means they are localised to the plasma membrane and function in phloem loading (Slewinski, 2009, Riesmeier et al., 1994). It was found that *AtSUT2* was necessary for loading sucrose into the phloem (Gottwald et al., 2000).

The nutrient transporter gene (*PsSUT*) was studied in this experiment to obtain a detailed account of the nutrient transport and their accumulation in field peas and process peas. It has been suggested that nutrient loading of seeds is a spatially and temporally dynamic process and it influences seed number at seed set and determines their final size (Zhang et al., 2007).

According to the phylogenetic tree, all isolated *PsSUT* gene family members studied in this thesis are plasma membrane located SUTs. Findings showed that the plasma membrane located SUTs are essential for phloem loading and normal pollen function (Sivitz et al., 2008). Evidences have shown that *AtSUT2* was essential for loading sucrose into phloem (Gottwald et al., 2000).

The expression of *PsSUT* genes varied between tissue types and developmental stages. Expression of both *SUT1a* and *SUT1b* was generally greater in pod walls than in seed tissues which is partially supported by the finding of Tegeder et al. (1999) that the signal strength of *PsSUT1* transcripts was weakest in seed coat tissues and the strongest expression was exhibited by developing flowers (Tegeder et al., 1999). It was also found that *PsSUT1* was expressed in non-seed tissues, which suggest wider role of this gene in sucrose transport (Tegeder et al., 1999). On the other hand, it was reported that *Phaseolus vulgaris SUT1*

(which has very close sequence homology to the *PsSUT1b*) showed very strong seed specificity, with high expression in cotyledons of developing bean seeds (Zhou et al., 2007). This difference in expression pattern between *PvSUT1* and *PsSUT1b* may suggest that these two species have developed a distinct mode of regulation.

The isolated *PsSUT2* had high sequence similarity with *AtSUC3*, which was found to be expressed in vegetative sinks as well as developing seeds (Zhang et al., 2007, Meyer et al., 2004). *PsSUT2* expressed in all developmental stages and tissue types and with a distinctively higher expression in the early developmental stages. Comparatively, the expression is generally higher in process peas. Interestingly, the expression is greater in the pod walls of field peas at 7 and 10 DAF. Subsequently, the expression decreases during later stages of seed development. This differential expression of *SUT* genes in developing seeds can be explained by the general rule that dominant sinks for nutrient loading shift from maternal tissues early in development (e.g. pod wall and seed coat), to filial tissues during later stages of development (Zhang et al., 2004, Zhang et al., 2007).

The sucrose facilitator gene, *SUF1*, exhibited very strong tissue specificity, as it was expressed more highly in the seed coat and seed tissues than in pod walls. This result agrees with those of Zhou *et al.* (2007), where they found that *PsSUF1* expression was strongest in cotyledons and coats of developing pea seeds (Zhou et al., 2007).

### **3.4.5 Identification, characterisation and expression of amino acid permease (AAP) transporter gene**

Arabidopsis contains a family of eight AAP members (*AtAAP1-8*), which show spatial and temporal expression (Fischer et al., 1995). It was found that *AtAAP1* and *AtAAP8* are involved in amino acid delivery to seeds during reproductive phase and also *AtAAP8* plays an important role in amino acid uptake into endosperm at early embryo stage (Schmidt et al., 2007). However, this member is also expressed in vasculature, presumably the phloem (Tegeder and Ward, 2012) of siliques, stem and other organs (Okumoto et al., 2002a).

According to Tegeder and Ward (2012), the identified AAP gene family members are grouped into four main clusters (1, 2, 3 and 4). Cluster 1 contains proteins from monocots

and eudicots. *AtAAP7* was grouped into this cluster. All the isolated *PsAAP7* gene family members were grouped together with *AtAAP7* and *CaAAP7*. The specific function of Cluster 1 members is still unknown, but they are important for seed plants since they are found in both monocots and dicots (Tegeder and Ward, 2012).

Cluster 3A contains *AtAAP2*, 3, 4 and 5 along with legume AAPs. The phylogenetic analysis of *PsAAP* gene family members revealed that the isolated *PsAAP2* (2a- 2e) and *PsAAP3* (3a and 3b) align with these members. The isolated *PsAAP2a*, *PsAAP2b*, *PsAAP2c*, *PsAAP2d*, *PsAAP2e* formed a clade with *AtAAP2*, *AtAAP4* and legumes *MtAAP*, *PsAAP2*, *CaAAP2* and *GmAAP2*. The *AtAAP2* and *AtAAP4* grouped together with high sequence similarity similar to the observation reported by (Okumoto et al., 2002a). Cluster 3A members function in loading of amino acid into phloem. In Arabidopsis, *AtAAP3* function was found to be restricted to the root (Okumoto et al., 2004), while *AtAAP5* functions in amino acid import into companion cells of root and leaves (Tegeder and Rentsch, 2010). Both *AtAAP2* and *AtAAP4* play an important role in leaf phloem loading (Zhang et al., 2010).

The isolated *PsAAP1*, *PsAAP6c*, *PsAAP6d* and *PsAAP8* were grouped with *AtAAP1*, 6 and 8 which come under Cluster 4B. Studies revealed that *AtAAP6* was localised to the leaf xylem parenchyma, to play a role in xylem- phloem transfer (Okumoto et al., 2002b). Evidence showed that both *AtAAP1* and *AtAAP8* were involved in seed loading, rather than phloem loading of amino acids (Sanders et al., 2009, Schmidt et al., 2007).

The *PsAAP1* and *PsAAP2* was isolated by Tegeder et al. (2000) from pea cotyledon cDNA library. Through northern blotting it was observed that *PsAAP1* expressed in pea cotyledons and strongest expression was noticed in stems and weak in sink leaves, whereas *PsAAP2* was not detected throughout the seeds (Tegeder et al., 2000). It was found that the amino acid transporter *AtAAP2* was localised in the phloem (Hirner et al., 1998). (Okumoto et al., 2002b). This is supported by another study by (Hunt et al., 2010). Large amounts of proteins are accumulated in cotyledons during legume seed development and amino acids necessary for the storage are imported into cotyledons from seed apoplasm (Tegeder et al., 2000).

Evidence have shown that the identified AAP gene family members are classified into four main clusters and have different functions (Tegeder and Ward, 2012). The isolated PsAAP gene family members studied in this experiment come under Cluster 1, 3A and 4B. Members of Cluster 3A are essential for phloem loading along the transport path (Zhang et al., 2010, Hirner et al., 1998) and are involved in amino acid import into the sieve element/ companion cells complex in legumes (Tegeder and Ward, 2012). Members of Cluster 4B play main role in seed loading, rather than phloem loading of amino acids (Sanders et al., 2009).

From the results of the phylogenetic analysis, it was revealed that the *PsAAP1* (Accession code: AY956395) – a sequence previously submitted to GenBank by Tegeder et al. (2000) is most similar to *AAP3* in other species. However, since the *PsAAP1* sequence identified by Dr Song is most similar to *AAP1* sequences in other species, it was decided to update the nomenclature and refer to this as the ‘real’ *PsAAP1*. Hence, it is important to note that the *PsAAP1* referred to in previous publications by (Tegeder et al. (2000), Tegeder et al. (2007)) is actually *PsAAP3*, and is shown as *PsAAP1[3]* in this study. *PsAAP3* or *PsAAP1[3]* is found to be a member of Cluster 3A, and functions in phloem loading, whereas *PsAAP1* comes under Cluster 4B which functions in seed loading.

Tegeder

*et al.* studied the expression of *PsAAP1[3]* and *PsAAP2* in two different experiments using a combination of northern blot analysis, *in situ* hybridisation and RT-qPCR approach. They found that both gene family members were expressed throughout the Bohatyr plants (field peas), but the transcript levels of *AAP1[3]* were highest in source leaves and pod walls compared to stems, roots, flowers and seeds, whereas *AAP2* transcript levels were expressed in all organs almost in same amount ( et al., 2000). The expression of *AAP1[3]* was strongest during the seed filling phase, while *AAP2* expression was detected during the early stages of seed coat development. Thus, the expression pattern of *PsAAP3a* in the present study is similar to that observed in the experiments carried out by Tegeder *et al.*, i.e. high *AAP1[3]* expression in pod walls. However, this gene family member showed strong expression during later stages of seed development as well.



### 3.4.6 Identification, characterisation and expression of *PscwINV* gene

When the phylogeny was done, different *cwINV* genes branched into 4 main clades. The isolated *PscwINV* gene family members were located in most of the clades. An isolated member *PscwINV240* was grouped with *VfcwINV1* which showed sequence similarity with *AtcwINV2* and 4. Three of the isolated members, *PscwINV415*, 285 and 681 formed a cluster with *MtcwINV5*. Another member *PsINV448* showed sequence similarity with *VfcwINV2*, whereas, *PsINV320* showed sequence similarity with *MtcwINV1*.

Expression of *cell wall invertase* has been observed in many systems (Roitsch and González, 2004), including developing fruit (Hayes et al., 2007) and seed (Weber, 2005). Importantly, elevation of the endogenous *cell wall invertase* activity significantly increased seed weight and fruit sugar hexoses in transgenic tomato and grain size in rice respectively, either by silencing its inhibitory protein (Jin et al., 2009) or over-expressing the proteins with its own promoter (Wang et al., 2008). These findings show that the development of seed and fruit is limited by *cell wall invertase* activity in these crops.

Weber et al. (1995) studied the molecular physiology of photosynthate unloading and partitioning during seed development of fava bean and found that during the pre-storage phase, there were high levels of hexoses in the cotyledons and the apoplastic endospermal space which correlated with high levels of *cell wall invertase* in the seed coat. During early stage, seed coat development is dominant, whereas the embryo is still undifferentiated. Therefore, the specific expression of *VfCWINV1* was important to provide the sink strength and to supply hexoses for seed coat development (Weber et al., 1995).

As suggested in several references, the isolated *PsINV* gene family members exhibited higher expression during early stages of seed development and also in the seed coats. It was observed in several plant species that an increase in invertase activity occurred in response to hormones such as gibberellins (Wu L, 1993) or cytokinins (Ehneß and Roitsch, 1997). It was not clear whether these effects were due to direct regulation of invertase genes by plant hormones or via stimulated cell proliferation creating new sinks for sucrose.

It was found that *VfCWINV1* was highly expressed in the seed coats (Weber et al., 1996), which is in agreement with the data shown in Figure 3.19H. The isolated gene family

member PsINV240, which shows sequence similarity with *VfCWINVI* in phylogenetic tree, was highly expressed in seed coats. This data also showed that most of the isolated pea invertase gene family members are up-regulated during very early stages of seed development and there was limited expression during later stages.

### 3.5 Summary

The data in this chapter provided the baseline data against which the transgenic plants over-expressing SUT and/or AAP transporter genes could be assessed. During development of seed, field pea and process pea showed differences in the nutrient content as they developed. It was evident from the data that seed coat and cotyledon have different nutrient contents at different stages, for example, cotyledon of both process pea and field pea contain starch during maturation, whereas seed coats did not have detectable amounts of starch at maturity.

Some difference between the field peas and process peas in gene expression were evident from very early stages of seed development. The expression of cytokinin regulatory genes increased at around 5 DAF. Noticeably, the expression pattern differed in field peas and process peas. The expression of the cytokinin biosynthetic genes increased in the pod walls of field peas at 5- 10 DAF whereas these genes have shown higher expression in the seeds of process peas. Concomitantly, the sucrose transporter genes were expressing at similar stages providing metabolites for the energy requiring process of cell division, differentiation and growth. Additionally, the *cwINV* genes showed elevated expression during early stages of development, which can again be related to cytokinin regulatory genes and *SUT* genes because as the cell division occurs or sink size increases, the imported sucrose needs to be cleaved and this is done by *cwINV* genes.

The activity of cytokinin regulatory genes and *cwINV* could be indirectly affected by activity of the nutrient transporters such as SUT and AAP. Hence, it is important to determine whether the transgenic plants over-expressing *PsSUT1* and *PsAAP1* have any effect on these genes, thereby affecting sink size and sink strength leading to increased yield.

## **CHAPTER 4**

### **Effect of ectopic expression of transporter genes in seed development**

#### **4.1 Introduction**

Seed development depends on a co-ordinated supply of nutrients especially carbohydrates and nitrogen to the developing embryo and storage tissues. During early embryogenesis, nutrients are mainly consumed by the growing embryo. Later in seed biogenesis, nutrients are used for the production of storage compounds like starch, oil and storage proteins. Transporters are important contributors in seed development, as they play a crucial role in nutrient transport.

The transgenic plants used in this chapter were previously developed lines of process and field pea, over-expressing a pea sucrose transporter (*PsSUT1*) and/or amino acid permease (*PsAAP1*) under the control of either 35S CaMV promoter or a tissue specific promoter (*AtAAP1*) (Table 2.1). The effect of the transgenes on expression of family members and on *cwINV*, *IPT* and *CKX* gene family members were examined.

#### **4.2 Materials and methods**

The materials and methods used in this chapter are outlined in Chapter 2.

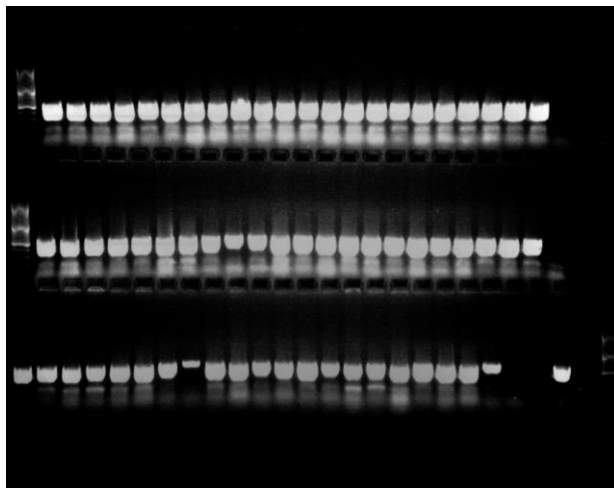
## 4.3 Results

### 4.3.1 Identification of double homozygotes

Figure 4.1 shows an example of a homozygote line that contains *PsAAP1* gene. First and second rows show that all 22 samples were positive whereas, the third row shows that one seed sample is negative for the particular gene. Twenty double homozygote seed lines were identified following seed DNA extraction, followed by PCR. The identified seed lines were:

- 1) MT11/MT1 - 19 seed lines tested; 4 were homozygous.
- 2) MT11/MT2 - 14 seed lines tested; 3 were homozygous.
- 3) MT2/MT11 - 11 seed lines tested; 3 were homozygous.
- 4) MT11/MT3 - 8 seed lines tested; 2 were homozygous.
- 5) MT3/MT11 - 8 seed lines tested; 2 were homozygous.
- 6) MT11/MT4 - 8 seed lines tested; 3 were homozygous.
- 7) MT4/MT11 - 8 seed lines tested; 3 were homozygous.

Those seed lines selected to be grown in the Biotron are listed in Table 2.1.



**Figure 4.1:** Gel picture showing PCR testing for MT1 gene.

### 4.3.2 Morphological and developmental differences between wild type peas and transgenic peas

To analyse the phenotypic characters of parental and transgenic peas, the plants were grown in the Lincoln University Biotron. Six sets of double homozygote seed lines were grown along with three single homozygotes (the parental lines) and two wild type pea cultivars (process pea and field pea). During development, the parental lines did not show any difference in morphology, although 5156, one of the double homozygote lines, was taller and had longer internodes.



**Figure 4.2: Images showing wild type and transgenic plants growing under controlled conditions.** A) Seedlings are starting to grow, which clearly shows that there were eleven plants in a tray and two trays for one seed line. B) Fully grown plants in which the taller plants are line 5156.

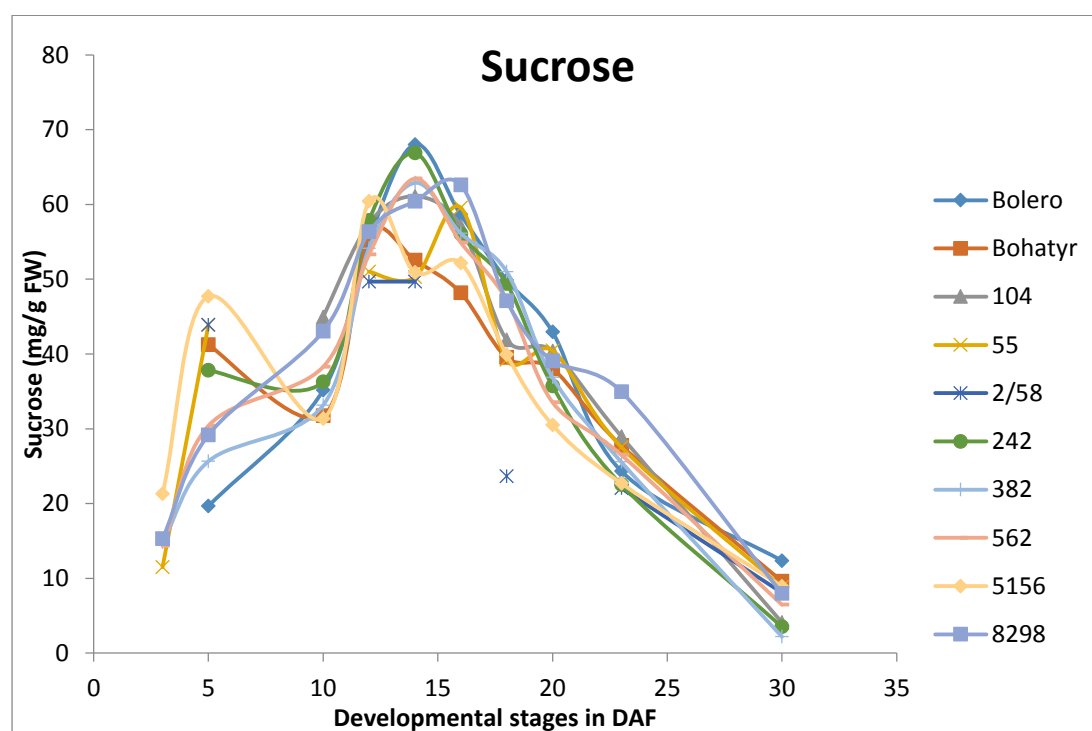
The results showed that there was a significant increase in the total number of seeds in double transgenic lines when compared with their parental wild type (Table 1.1). Out of six double homozygotes, two lines showed a consistent increase in seed number (Table 1.1). Those two lines were 382 and 5156.

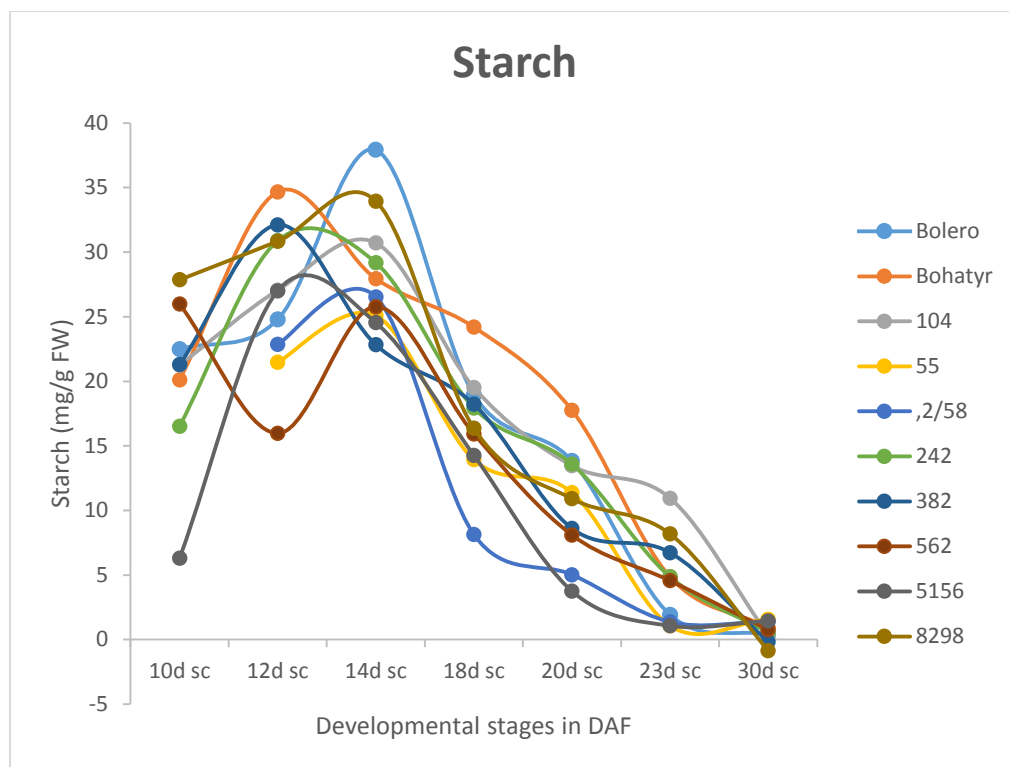
### 4.3.3 Sucrose and starch analysis

Figure 4.3 shows the sucrose and starch content in the seed coats of the developing seed of field pea, process pea and their transgenic lines. The sucrose content was greatest in the seed coats of process pea (Bolero) and a double homozygote line 242 and at a maximum at 14 DAF, while that of field pea lines was at a maximum at 12 DAF. At maturity, the amount of

sucrose is much reduced in both WT and transgenic lines. But comparatively, Bolero had the highest sucrose content at 30 DAF and line 382 had the lowest sucrose content. Noticeably, the parental line 2/58 showed very low sucrose content at 18 DAF. The double transgenic line 8298 had high sucrose content at most of the stages. On the other hand, line 5156 showed particularly low amount of sucrose at 14d.

The starch present in the seed coats of field and process peas was highest at 12 and 14 DAF respectively. At the later stages (18, 20, and 23 DAF), the seed coats of Bohatyr still contained some starch.





**Figure 4.3: Sucrose and starch content in the seed coats of developing seeds.** Sucrose and starch content was measured as mg/g of FW and was plotted against the developing stages. 5d samples were the whole seed, whereas from 10d, seed coat removed from the cotyledon was used as the sample.

### 4.3.3 Gene expression analysis

All the melting and amplification curves of different gene family members (GFM) are shown in Appendix Section 3.1.

#### 4.3.3.1 Expression analysis of *PsSUT* gene in transgenic and wild type peas

In this study, *PsSUT1a* primers should detect the *AtAAP1::PsSUT1* transgene. The transgene was detected, as shown by up-regulation of this gene family member in the transgenic lines. Among the process pea lines, both the double transgenics showed elevated *PsSUT1a* expression (Figure 4.4A). In the case of field pea lines, all the transgenic lines showed almost same level of expression, even though only 5156 carried the *PsSUT1* transgene. Seed line 5156 showed greater expression in very early stages (-1 and 0 DAF) and in pod walls of 7 DAF (Figure 4.4C). However, in the case of seed coats transgenic lines did not show consistently greater expression than wild type.

All the isolated *PsSUT* gene family members showed almost similar pattern of expression during seed development. However, sucrose facilitator (*PsSUF1*) was strongly expressed in seeds, unlike other gene family members which expressed highly in pod walls. Also, most of the gene family members were highly expressed in seed coats of developing cotyledons. Only *PsSUT10* showed low expression in seed coats.

*PsSUT1b* showed relatively similar expression as *PsSUT1a*, that is, greater expression during early stages, especially in pod walls (Fig. 4.4E, G). In the case of the seed coats, this gene family member showed much greater expression in all transgenic lines compared with wild type (Fig. 4.4F, H).

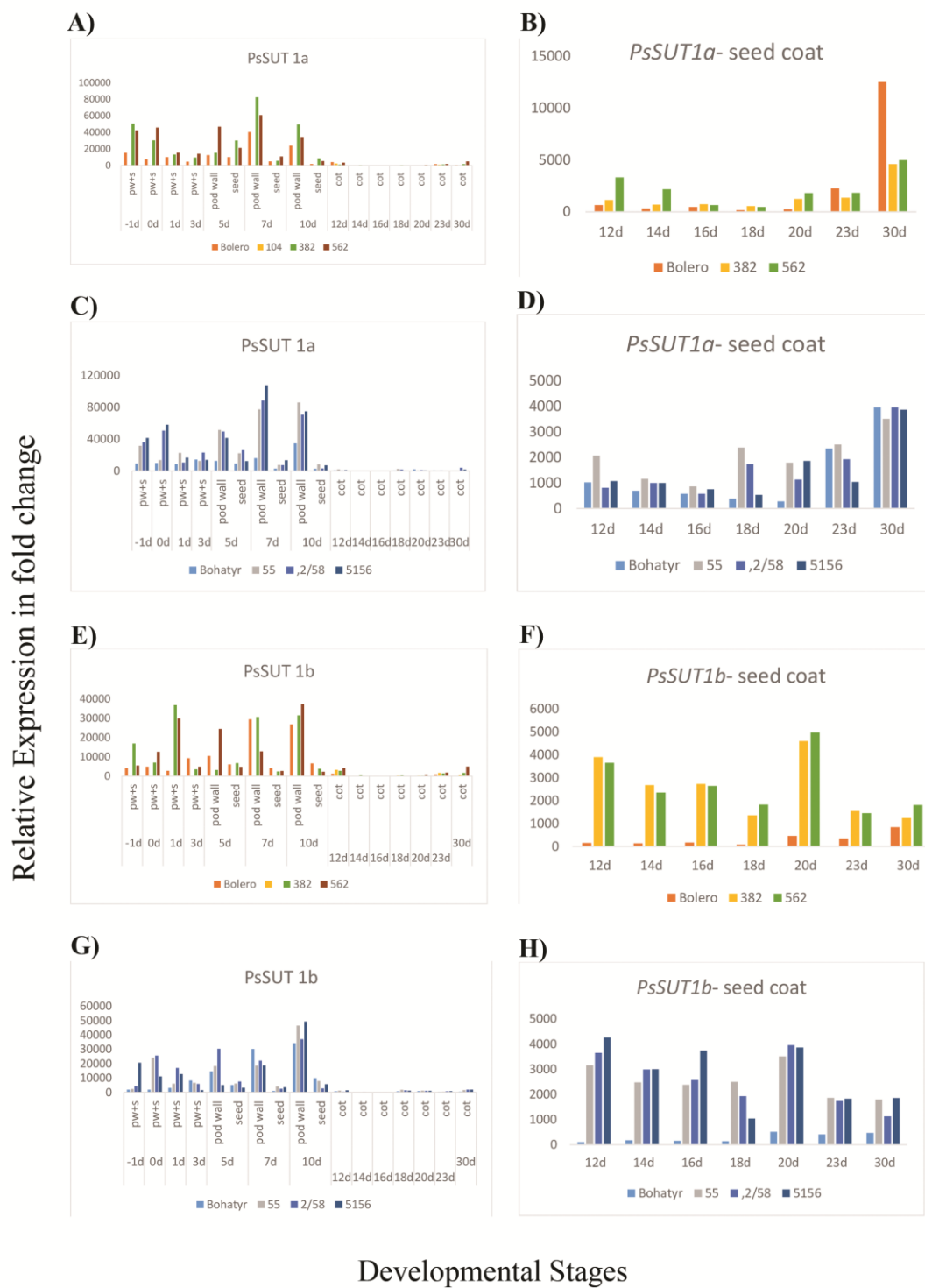
*PsSUT2* showed elevated expression during early stages of development and this GFM was expressed in pod walls as well as seeds. The Bolero transgenic lines 382 and 562 particularly showed elevated expression in pod walls and seeds at 5, 7 and 10 DAF (Fig.4.4I). In the case of field pea lines, the parental lines (2/58, 55) showed elevated expression most of the time, compared with the double homozygote line 5156 (Fig. 4.4K). In the case of seed coats, *PsSUT2* showed greater expression in all the transgenic lines compared with their wild type. Especially at 14, 16 and 18 DAF this gene family member was highly expressed in seed coats and the expression decreased gradually in all lines (Fig. 4.4J, L).

*PsSUT10* showed comparatively much less expression throughout the development, though early stages showed greater expression than the later stages. This GFM had greater expression in transgenic lines than the WT. And also, this GFM showed hardly any expression in the cotyledons. Expression of *PsSUT10* in seed coats was much less than other GFM. (Fig. 4.4N,P).

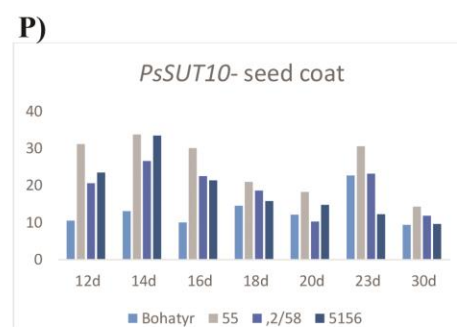
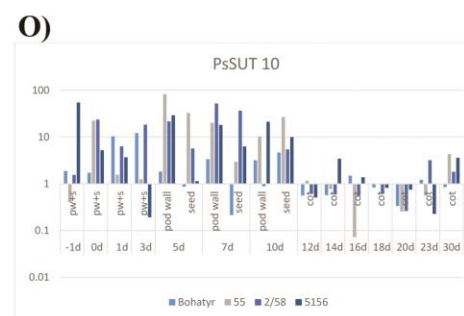
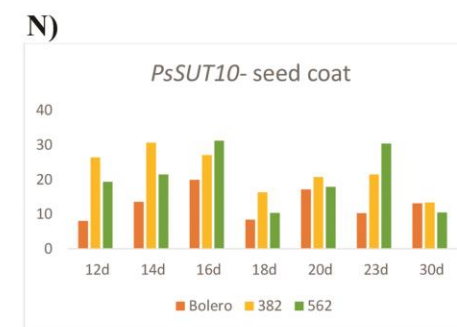
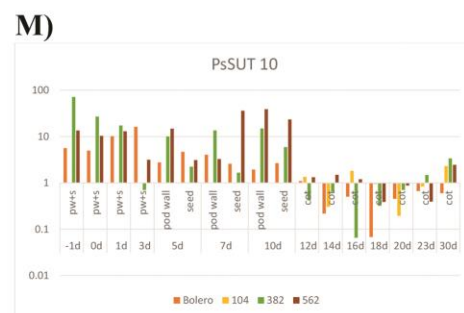
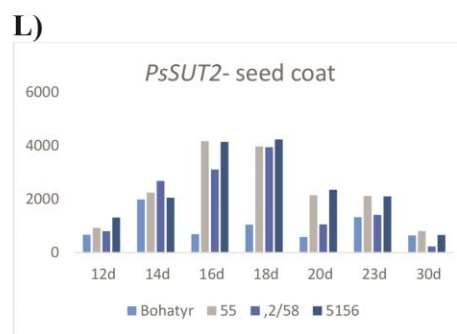
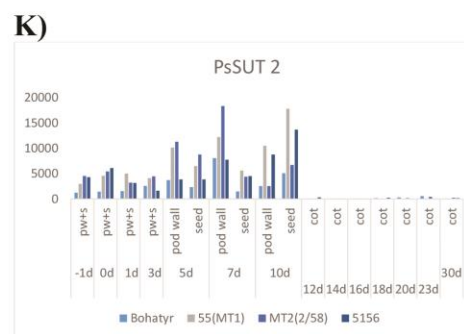
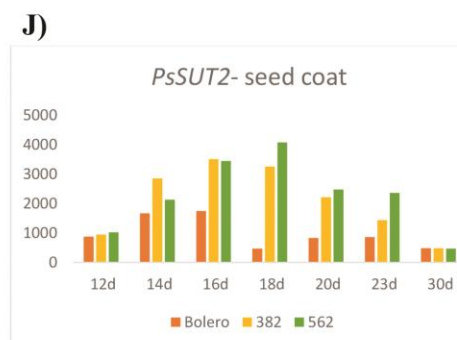
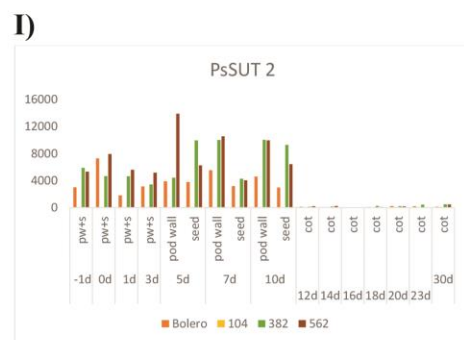
*PsSUF1* was expressed in young seeds of both WT and transgenic plants. Line 382 showed the maximum expression at early stages, whereas line 562 had greater expression at the later stages, among process peas (Fig 4.4Q). On the other hand, the parental line (2/58) and 5156 showed greater expression at early stages of development (Fig 4.4S). But in seed coats, this gene was highly expressed during the cotyledon expansion stage (12, 14 DAF). The greatest expression was shown at 12 DAF. Then the expression decreased and was much reduced during the maturation phases (20, 23 and 30 DAF). Also, transgenic lines showed greater expression than the WT (Fig 4.4 R, T).



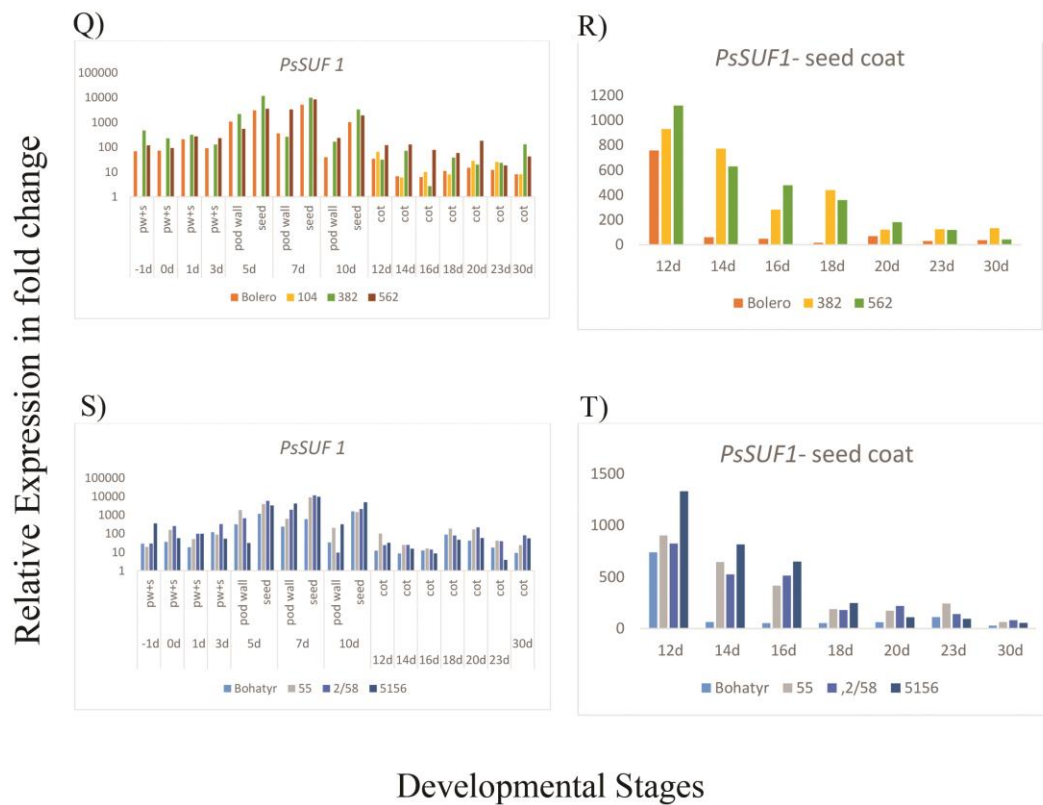




Relative Expression in fold change



Developmental Stages



**Figure 4.4: Relative expression of *PsSUT1a*, *b*, *2* and *10* and *PsSUF1* gene family members in developing seeds and seed coats of WT and transgenic lines.** A,C) *PsSUT1a* in process pea varieties and field pea varieties. B,D) *PsSUT1a* in seed coats of process pea varieties and field pea varieties. E,G) *PsSUT1b* in process pea varieties and field pea varieties. F,H) *PsSUT1b* in seed coats of process pea varieties and field pea varieties. I,K) *PsSUT2* in process pea varieties and field pea varieties. J,L) *PsSUT2* in seed coats of process pea varieties and field pea varieties. M,O) *PsSUT10* in process pea varieties and field pea varieties. N,P) *PsSUT10* in seed coats of process pea varieties and field pea varieties. Q,S) *PsSUF* in process pea varieties and field pea varieties. R,T) *PsSUF* in seed coats of process pea varieties and field pea varieties.

#### **4.3.3.2 Expression analysis of *PsAAP* gene in transgenic and wild type peas**

In this study, *PsAAP3a* primers should detect the transgene, labelled by Plant & Food Research Institute as *PsAAP1* (i.e., 35S::PsAAP1).

Since, *PsAAP1/3* is the transgene in this study, *PsAAP3a* is the most highly expressed gene family member of *PsAAP*. The transgene showed elevated expression in the younger stages, especially in the pod walls (Fig 4.5 A, C). In the case of process pea varieties, the double transgenic lines showed a much higher expression than the WT. But in the cotyledons, the expression decreased gradually and again at the final stages (20, 23 and 30 DAF) all the lines except the parental line showed elevated expression (Fig 4.5 A). Among the field pea lines, only one parental line (1/55) has 35S:: PsAAP1, which showed higher expression than others during early stages, but not later stages of seed development. The double transgenic line did not show much difference in expression compared with WT (Fig 4.6B).

In the case of seed coats, double transgenic lines 382, 562 showed greater expression than the WT (Fig 4.5 B). Noticeably, the expression was at the maximum at 18, 20 and 23 DAF. Among the process pea varieties, although both 382 and 562 have the transgene, line 382 showed greater expression than 562 in most of the stages. But at maturity, line 562 showed greater expression (Fig 4.5 B). But in case of field pea lines, the double transgenic line and the parental lines showed elevated expression, particularly at 20 DAF (Fig 4.5 D).

Gene expression of fifteen *PsAAP* gene family members was studied, out of which fourteen members showed a temporal expression pattern, whereas one member showed hardly any expression throughout seed development.

*PsAAP3b* showed an interesting expression pattern in the cotyledons of 12, 14 and 16 DAF. The gene family member is hardly expressed in the cotyledons at this stage, but the expression increased after that, and the highest expression was at 30 DAF (Fig 4.5 U,W). In the case of seed coats, the WT had hardly any expression until 23 DAF, whereas the double transgenic lines showed greater expression at 16, 18 and 20 DAF, and then the expression decreased gradually (Fig 4.5 V). Between field pea lines, the double transgenic line, 5156 showed greater expression than the parental lines until 20 DAF. Among the parental lines, 1/55 showed greater expression than 2/58 (Fig 4.5 X).

*PsAAP1* showed greater expression during early stages of seed development, specifically in the seeds. Also, double transgenic lines had greater expression than the WT. Among two transgenics, the line 382 showed a somewhat more elevated expression than 562 at most of the stages (Fig 4.5 E). This GFM was down-regulated in the cotyledons, from 12 DAF. But during storage phase, WT as well as transgenic lines showed expression in the cotyledons. Among the field pea lines, both single and double transgenic lines showed greater expression than the WT. Line 1/55 had greater expression than 2/58 at most of the stages. Similarly, during later stages of development, the expression was decreased and again at the maturation phase (18, 20 and 23 DAF) expression was increased (Fig 4.5 G).

In the case of process pea seed coats, the double transgenic lines showed greater expression than the WT. Also, the gene expression was maximum at 20, 23 DAF, and then decreased (Fig 4.5 F). Between the seed coats of the field pea lines, the WT showed greater expression than the transgenic lines at 12, 14 DAF, and then the expression decreased until 23 DAF. The double transgenic line, 5156 showed elevated expression than the parental lines in most of the developmental stages (Fig 4.5 H).

Three of the *PsAAP2* family members (2a, 2d and 2e) showed similar expression pattern, that is, higher expression during early stages of seed development than the later stages. Similarly, these gene family members exhibited elevated expression in pod walls (Fig 4.5 I, K, M, O, Q and S).

All three gene family members showed similar pattern of expression in the early stages, although *PsAAP2a* showed a slightly different pattern during later stages. Expression for this gene family member had decreased markedly in the cotyledons of 12 and 14 DAF, and then expression increased from 16 DAF. The parental line 104 showed almost similar or higher expression than the double transgenic lines (Fig 4.5 I). In the case of field pea varieties, the line 5156 showed greater expression than its parental line, 2/58 and the WT (Fig 4.5 K).

In the case of seed coats, the expression of *PsAAP2a* increased gradually and was the greatest at 16, 18 DAF, then again decreased. And also, the WT showed hardly any expression for this GFM. Among the double transgenics, line 382 showed greater expression than 562 in most of the stages (Fig 4.5 J). The field pea lines mimicked the same trend in the expression pattern. The expression of WT was negligible, compared with its transgenic lines. Interestingly, the

double transgenic line 5156 showed greater expression than the parental lines, 1/55 and 2/58 (Fig 4.5 L).

*PsAAP2d* showed high expression during the early stages of seed development, in both cultivars and their transgenics, though the expression decreased a little in the cotyledons of 12 DAF, then they showed a stable level of expression until 23 DAF. Again, at the final stage (30 DAF), all the lines showed an elevated expression (Fig 4.5 M and O). This gene family member did not show much difference between the parental lines and the double transgenic lines.

*PsAAP2e* maintained relatively similar level of expression throughout the development. However, it showed slightly elevated expression, specifically in the pod walls (Fig 4.5 Q and S). In both the pea cultivars, parental lines showed greater or similar expression to that of the double transgenics. This GFM was the least expressed one among AAPs in the seed coats (note the scale). The transgenic lines showed greater expression than the WT (Fig 4.5 Q). Among the field peas lines, the transgenic lines showed more elevated expression than the WT. The double transgenic line, 5156 showed greater expression than the parental lines in almost all stages (Fig 4.5 S).

*PsAAP6c* and *6d* showed different patterns of expression during the seed development. *PsAAP6c* was more highly expressed in the young seeds compared with pod walls (Fig 4.5Y, Aa), whereas, *PsAAP6d* showed greater expression in the pod walls (Fig 4.5 Ac, Ae). Transgenic lines showed greater expression than the WT for both the gene family members. The cotyledons of 12, 14 and 16 DAF showed less expression comparatively, then the expression tended to increase up to 30 DAF.

In the case of seed coats, the expression of *PsAAP6c* increased as the seed matured and then decreased gradually and was much less at 30 DAF. The transgenic lines showed more elevated expression than the WT (Fig 4.5 Z). Among the field pea lines, the WT had the greatest expression at 14 DF. Also, the expression pattern was similar as that of process pea lines. But at maturity, all the transgenic lines had less expression, whereas the WT showed elevated expression (Fig 4.5 Ab).

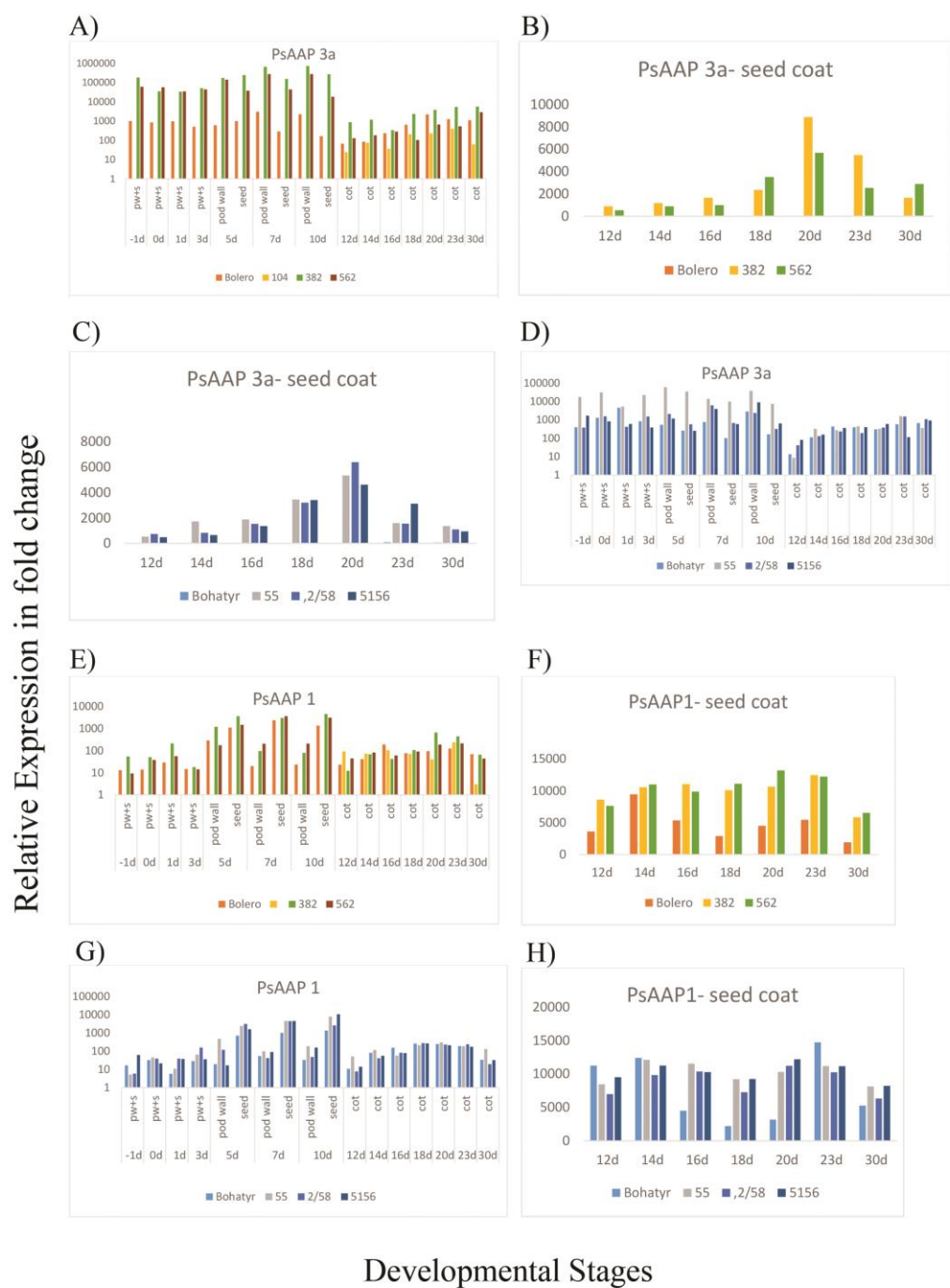
Expression of *PsAAP6d* was much less in seed coats in the beginning, then increased until 16 DAF and then decreased. Again, the expression increased to reach the maximum at 20 DAF.

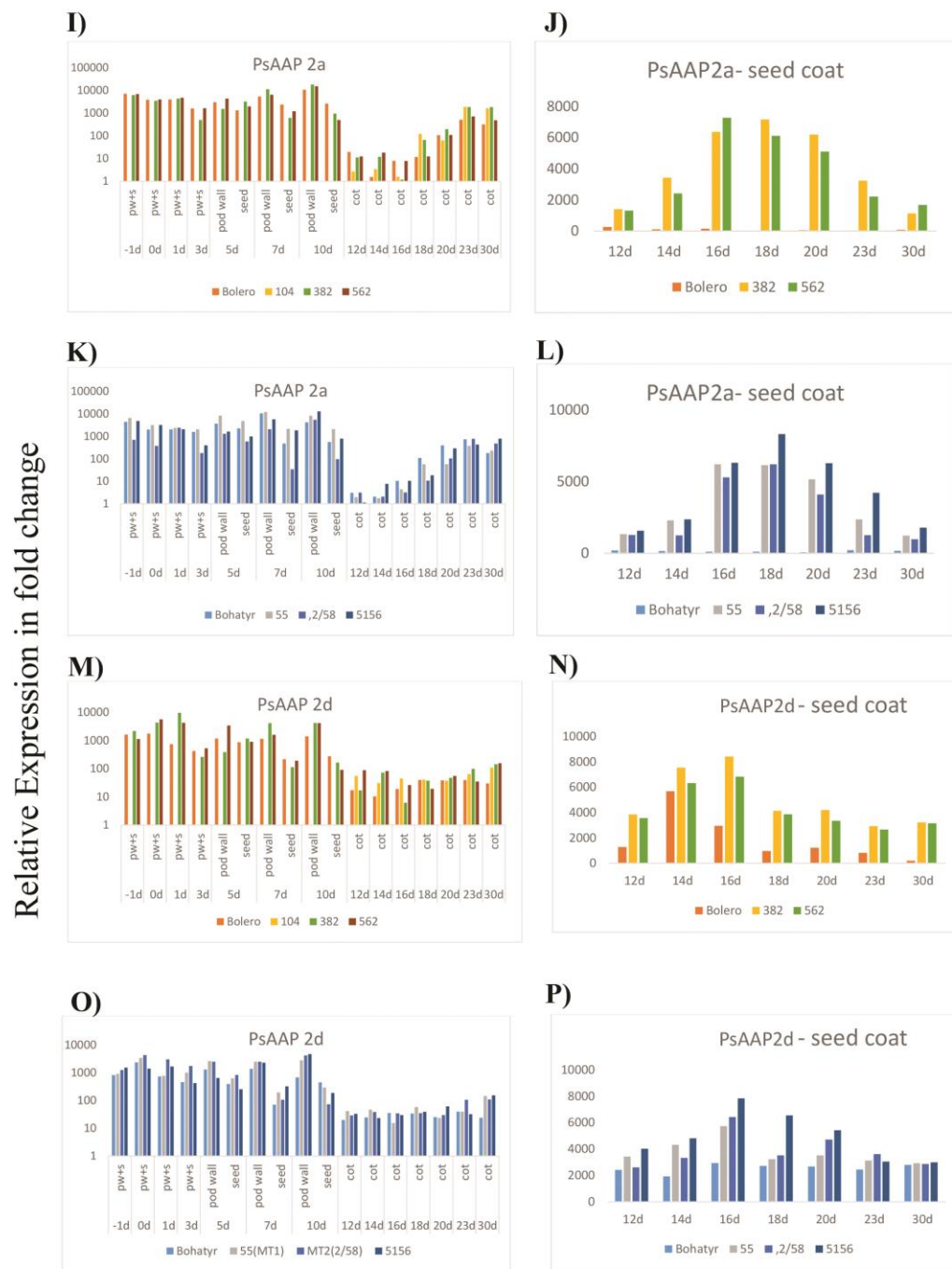
At maturity, only line 382 showed elevated expression (Fig 4.5 Ad). The expression pattern was similar in the case of field pea lines (fluctuated expression) (Fig 4.5 Af).

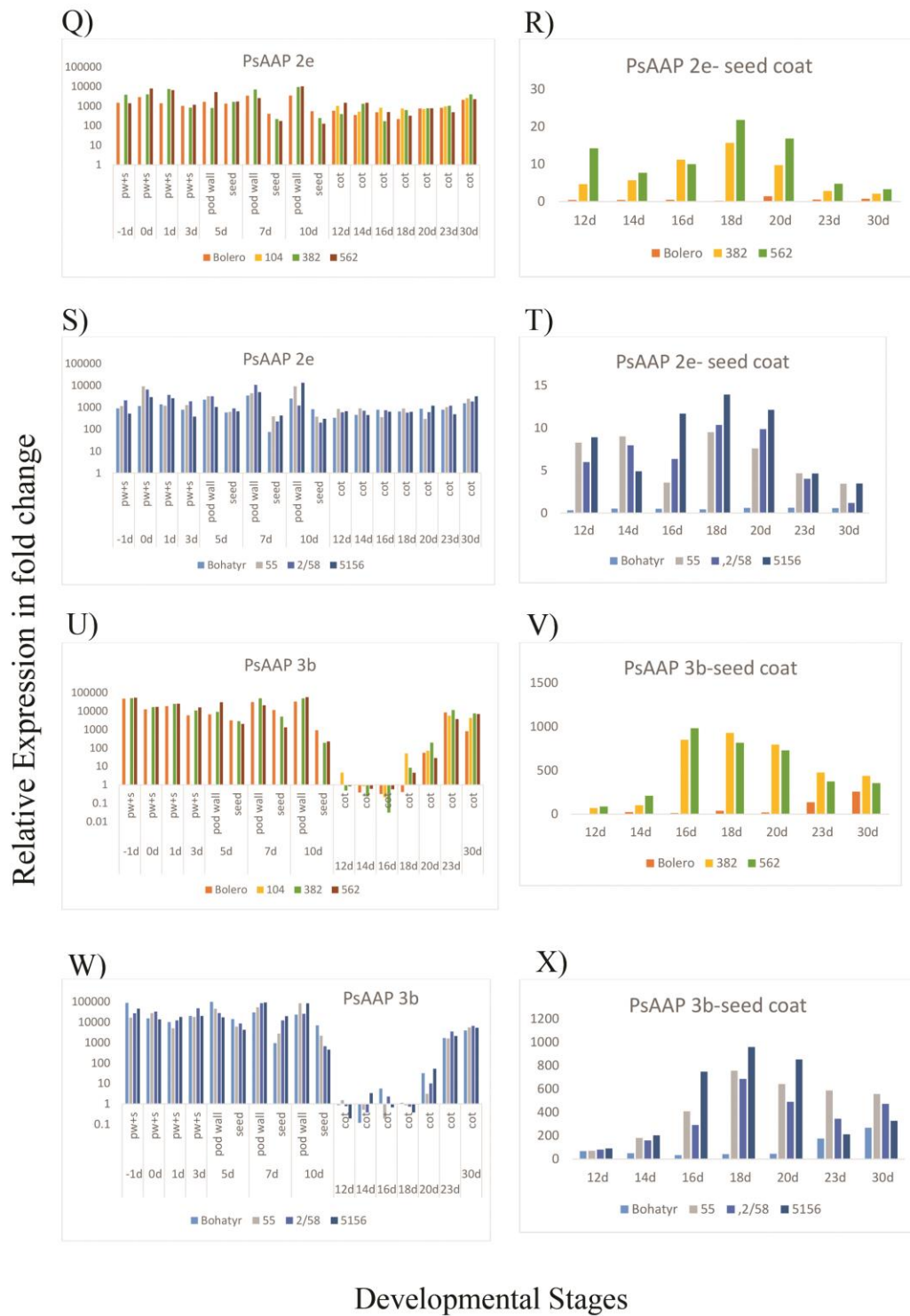
All the members of the *PsAAP7* family clusters showed almost similar pattern of expression. All the members showed elevated expression in the pod walls. However, 7b and 7c showed more similarity in the expression pattern. Both the members were highly expressed during the early stages then the expression was very low in the cotyledons of 12 and 14 DAF, then again it increased during maturation phase. The double transgenic lines showed higher expression than the parental lines, in the case of both process pea and field pea varieties (Fig 4.5 Ag, Ai, Ak, Am). Expression of *PsAAP7b* in seed coats was greater during early stages and decreased gradually. Transgenic lines showed more elevated expression compared with the WT (Fig 4.5 Ag). Expression pattern was similar in the case of field peas. Between the transgenic lines, the parental line, 1/55 showed greater expression than others in almost all developmental stages (Fig 4.5 Ai).

*PsAAP7e* showed low expression, compared with other members (note the fold change). However, the expression was fluctuating throughout the development in both WT and transgenic plants. Also, there was not much difference between the parental lines and the double transgenic lines (Fig 4.5 Ao, Aq). In the case of seed coats, the expression of this GFM was greater at 12- 16 DAF, then decreased. The expression was higher in the transgenic lines, compared with WT (Fig 4.5 Ap). On the other hand, the expression pattern varied among the field pea lines. The parental line, 1/55 showed greater expression at most of the stages. But interestingly, the WT (Bohatyr) had the greatest expression at 23 and 30 DAF (Fig 4.5 Ar).



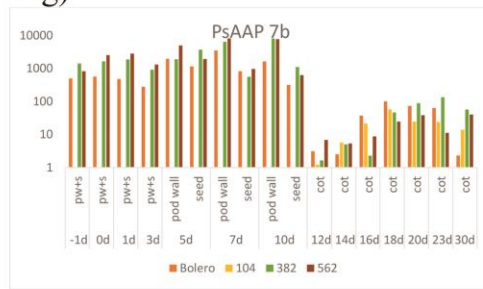




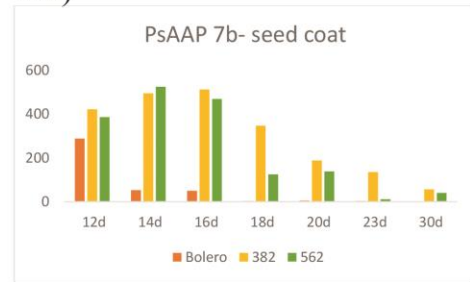




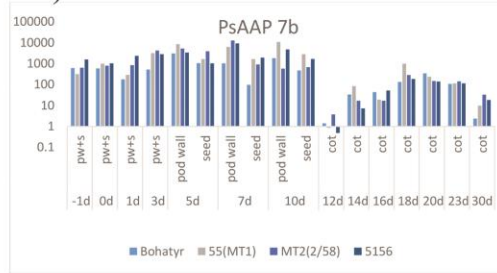
Ag)



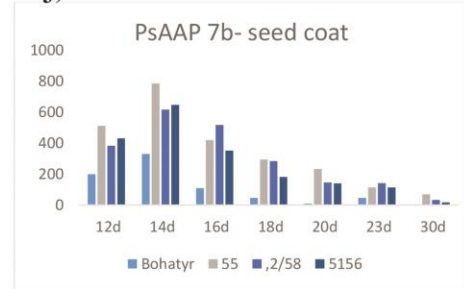
Ah)



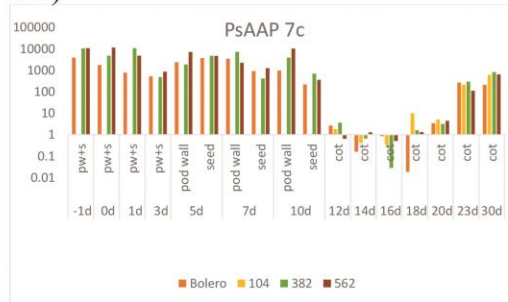
Ai)



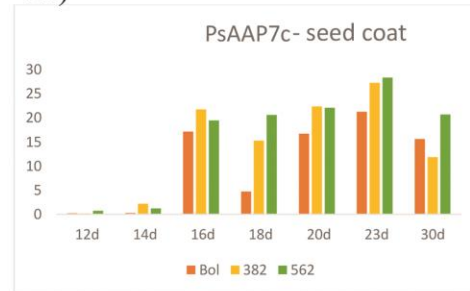
Aj)



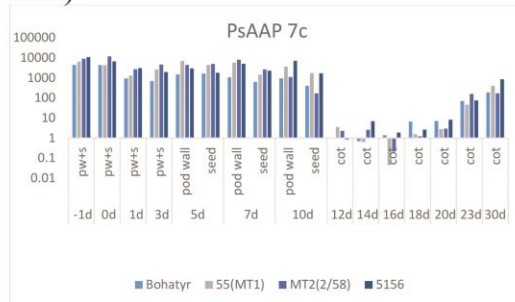
Ak)



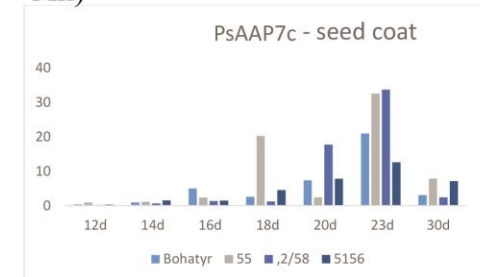
Al)



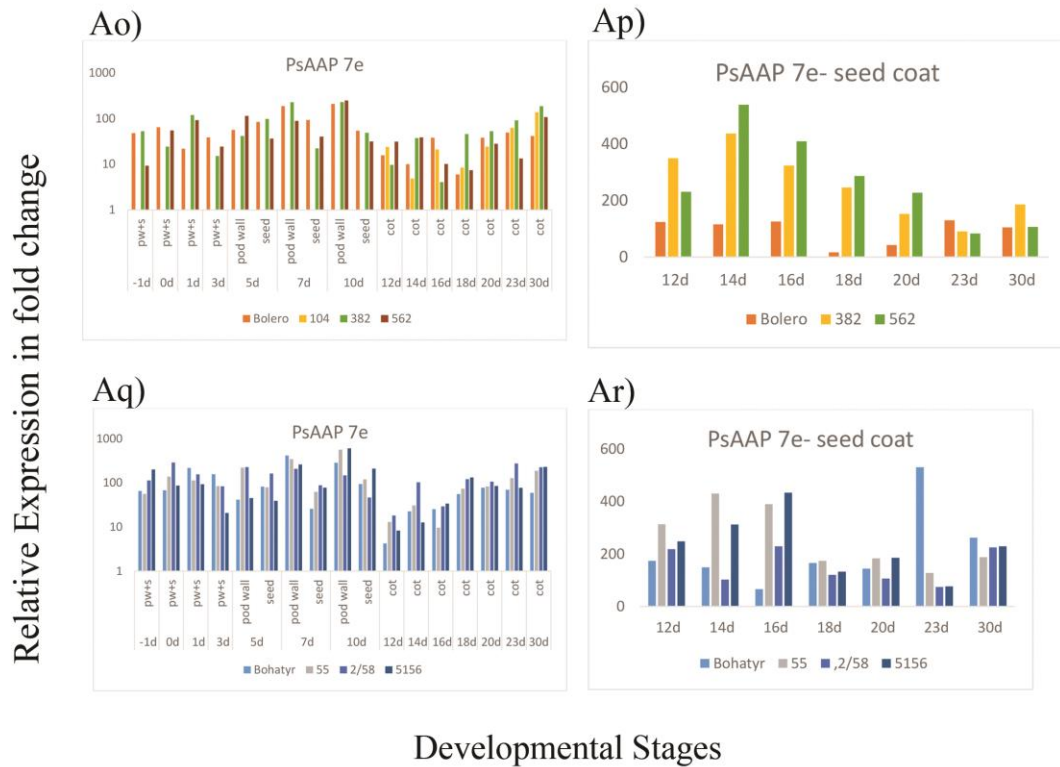
Am)



An)



Developmental Stages



**Figure 4.5: Relative expression of *PsAAP1*, 2, 3, 6 and 7 gene family members in developing seeds of WT and transgenic lines. A,C) *PsAAP1a* in process pea varieties and field pea varieties.**

#### 4.3.3.3 Expression analysis of *PsIPT* gene in transgenic and wild type peas

The relative expression of cytokinin biosynthetic genes was studied in process pea, field pea and their transgenics. Each graph either shows the gene expression of process pea and its transgenic lines, or field pea and its transgenic lines. Both wild type (WT) and transgenic plants show an elevated expression of *PsIPT2* during early stages of seed development, especially in pod walls.

Figure 4.6A shows the expression of process pea (Bolero) and its double homozygote lines, 382 and 562. Both the lines contain transgenes *AtAAP1::PsSUT1* and *35S::PsAAP1*.

Transgenic plants showed greater expression than wild type (WT) (Fig 4.6A) especially during early stages of development.

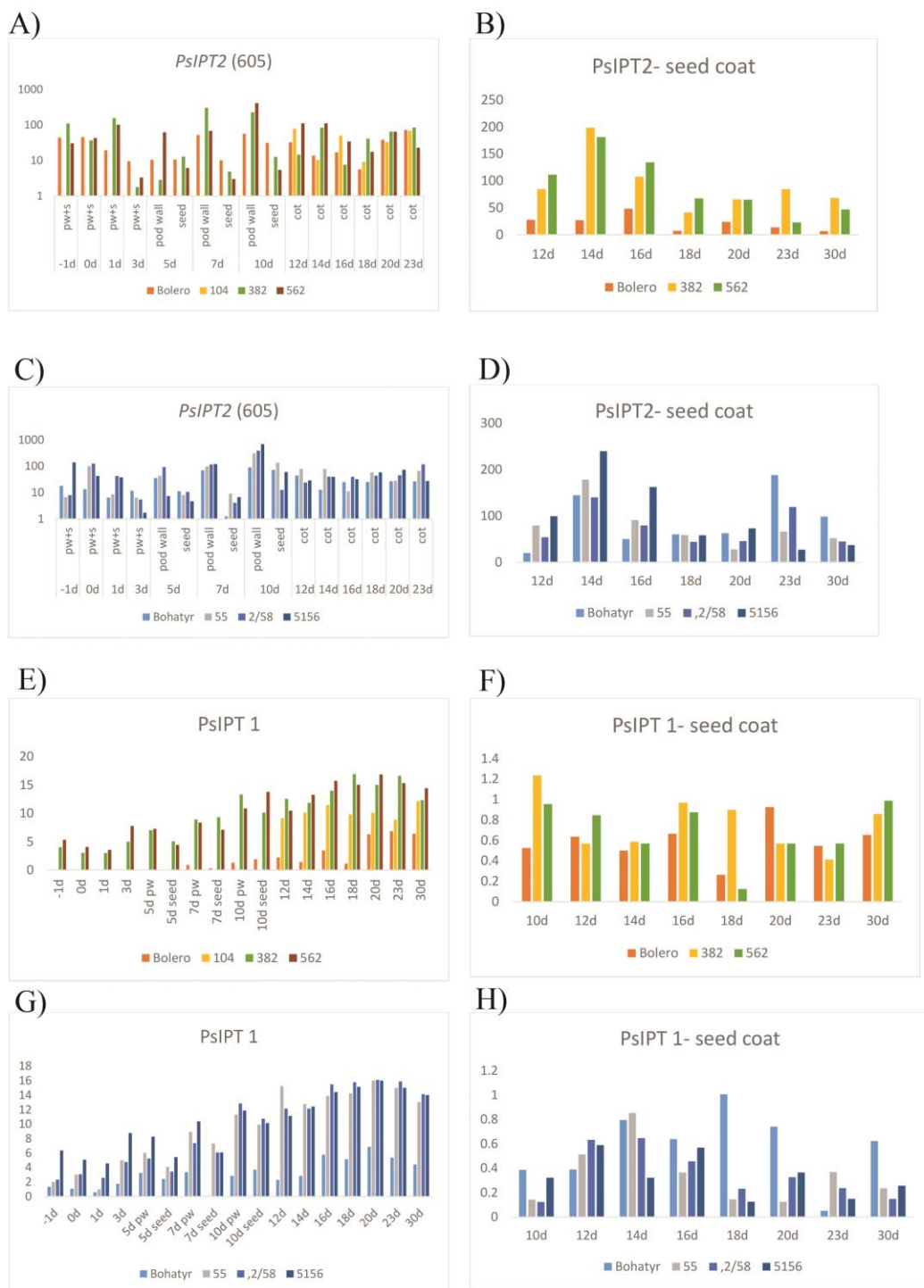
Figure 4.6 C gives the expression of field pea (Bohatyr), parental transgenic lines, 55 and 2/58, and the double homozygote line, 5156. All the transgenic lines showed elevated expression compared with the wild type (WT), for this gene family member. Again, expression was greater in pod walls, compared with seeds and cotyledons. Also, the greatest expression was shown by the double homozygote line at 10 DAF.

In the case of seed coats, the expression was greater at 14 DAF, then it decreased. Both the double transgenic lines showed almost similar level of expression at all stages, but at 23d line 382 was more elevated than 562 (Fig 4.6 B). Among field pea lines, the WT showed greater expression during the storage phase (20, 23d), whereas the double transgenic line 5156 showed more elevated expression during early stages (12- 20 DAF) (Fig 4.6 D).

Expression of *PsIPT1* was relatively much less than other GFM. The later stages of development showed greater expression than the early stages. And also, transgenic lines showed more elevated expression than WT at all stages (Fig 4.6 E). Field pea lines showed similar pattern of expression. During the early stages, line 5156 showed the greatest expression, but at later stages, parental lines and double transgenic lines had elevated expression (Fig 4.6 G).

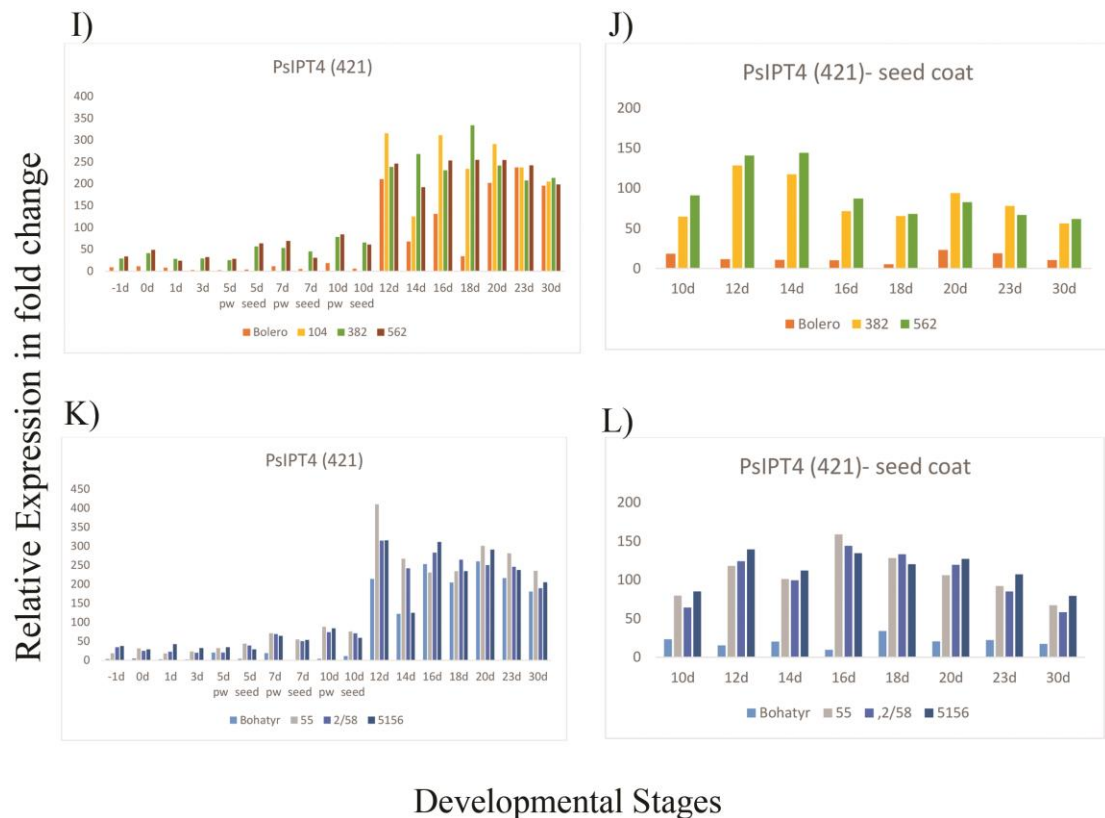
The expression was much less or negligible in the case of seed coats. The transgenic lines showed more elevated expression than the WT at most of the stages (Fig 4.6 F). But among field pea lines, the WT showed greater expression than the transgenic lines (Fig 4.6 H).

The expression of *PsIPT4* was greater during the later stages of development, relative to early stages of development. Transgenic lines showed more elevated expression than WT in all stages (Fig 4.6 I). Again, field pea lines showed similar expression pattern. The parental line 1/55 was more elevated than other lines in almost all stages (Fig 4.6 K). In the case of seed coats, both process pea and field pea lines showed similar expression pattern. Both varieties showed greater expression for the transgenic lines, compared with WT. The expression was more at 12, 14, and 16 DAF, then decreased. The double transgenic lines 562 and 5156 showed the highest in two different varieties (Fig 4.6 J, L).



Developmental Stages





**Figure 4.6: Relative expression of *PsIPT1*, 2 and 4 gene family members in developing seeds of WT and transgenic lines.** A,C) *PsIPT2* in process pea lines and field pea lines. Data are means of relative mRNA levels in fold changes detected using three technical replicates. X-Axis is the developmental stages from -1 to 30 DAF, in which -1 to 3 DAF is the whole pod with seeds, 5 to 10 DAF gives separated seeds from the pod wall, 12 to 30 DAF is dissected cotyledon without seed coat. Y-Axis is the relative expression in fold change. Note: Line 104- only cotyledon material was available for analysis.

#### 4.3.3.4 Expression analysis of *PsCKX* gene in transgenic and wild type peas

All of the isolated *PsCKX* gene family members showed spatial and temporal expression patterns. *PsCKX1* was strongly expressed in the young seeds. Transgenic lines showed greater expression than WT (Fig 4.7A). Between the transgenic lines, 382 generally had greater expression than 562. In the case of field pea varieties, both single and double homozygote lines showed increased expression compared with the WT (Fig 4.7C). The seed

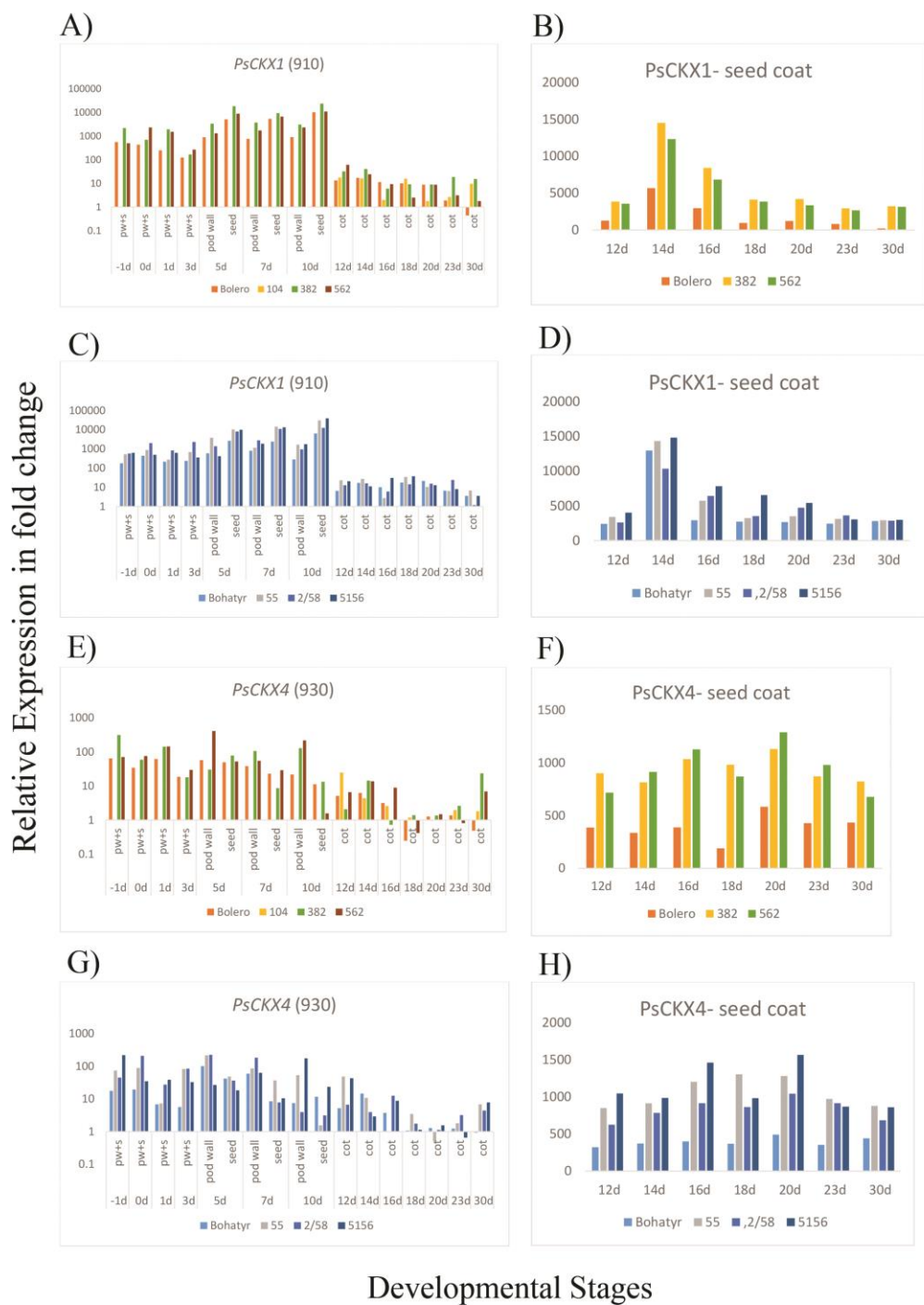
line 5156 showed the highest expression and the parental line 1/55 also showed elevated expression. In the case of seed coats, transgenic lines showed greater expression than wild type. Temporal expression was seen in seed coats. The highest expression was shown at 14 and 16 DAF in all the lines. Then the expression decreased and was steady at the maturation phase.

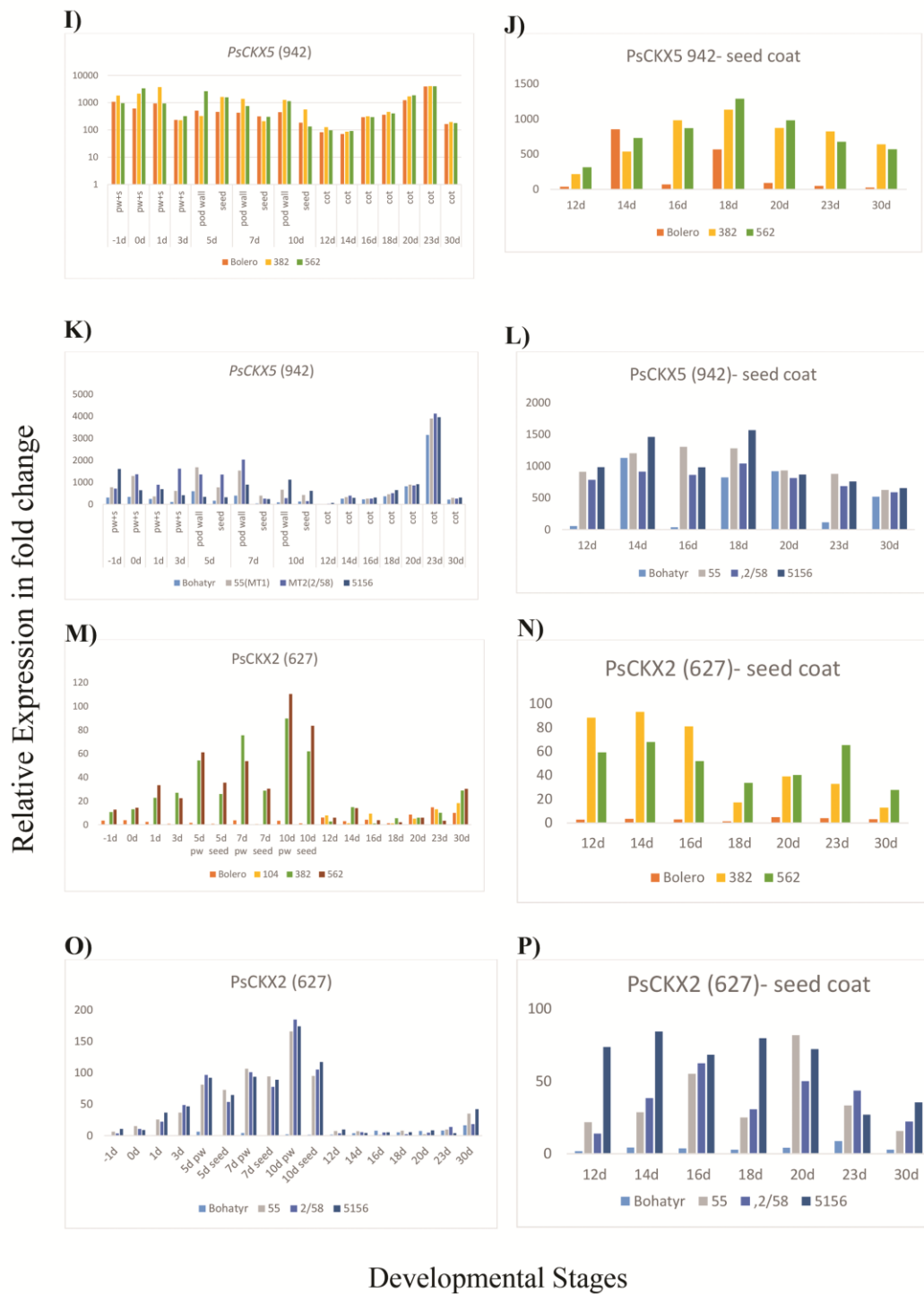
*PsCKX4* had elevated expression pattern at very early stages (-1, 0, 1 DAF) as well as in pod walls (Fig 4.7 E,G). Both the double transgenic lines showed greater expression than its WT, process pea. Figure 4.7 G shows the expression levels in field pea and its transgenic plants. The WT showed very little expression, when compared with the transgenic lines. The double homozygote seed line, 5156 showed elevated expression in very young pods (-1 DAF) and also in pod walls of 7 and 10 DAF. Expression of this gene family member was very low during the later stages of seed development, in WT and transgenic lines. This gene family member showed great expression in developing seed coats too. All the transgenic lines showed greater expression than wild type. Also, this gene family member was highly expressed at 14, 16, 18 and 20 DAF. Then the expression decreased during the final stages of seed development.

*PsCKX5* was highly expressed at the very early stages of development and also at very later stages. This GFM showed greater expression in pod walls than seeds. Also, the transgenic lines showed more expression than WT (Fig 4.7 I). Among the field pea lines, the parental line 2/58, and double transgenic line 5156 showed greater expression. The expression pattern was same as that of process pea lines, except for the strongly elevated expression at 23 DAF. In the case of seed coats, WT showed less expression than transgenic lines, even though Bolero showed elevated expression at 14 and 18 DAF. The gene expression was maximum at 18 DAF and then decreased (Fig 4.7 J). Between field pea lines, the double transgenic line 5156 had greater expression than others in most of the stages. Between the parental lines, 1/55 showed greater expression than 2/58 (Fig 4.7 K).

Expression of *PsCKX2* was greater during early stages of development than the later stages. And also, the expression was more in pod walls than seeds. Transgenic lines showed greater expression than WT. At maturity (30d), all the lines showed some expression (Fig 4.7 M). Field pea lines showed similar expression pattern. Both the parental lines showed great expression during early stages of development. The double transgenic line 5156 showed

greater expression in seed at 10 and 30 DAF (Fig 4.7 O). The expression pattern was similar in the seed coats of process peas and field peas. Expression was more in transgenic lines compared with WT. Process pea lines showed maximum expression at 12, 14 and 16 DAF, and then the expression decreased. And then line 562 showed elevated expression at 23 and 30 DAF (Fig 4.7 N). Among field pea lines, the double transgenic line 5156 showed the greatest expression in almost all stages (Fig 4.7 P).





**Figure 4.7: Relative expression of *PsCKX1*, 2 and 4 gene family members in developing seeds of WT and transgenic lines. A,B,C,D) *PsCKX1*, E,F,G,H) *PsCKX4*, I,J,K,L) *PsCKX5* M,N,O,P) *PsCKX2* in process pea and field pea lines.**

#### ***4.3.3.5 Expression analysis of PscwINV gene in transgenic and wild type peas***

Three of the isolated *PscwINV* gene family members (PsINV285, PsINV320 and PsINV448) exhibited greater expression during early stages of seed development but reduced expression during the maturation phase (Fig 4.8 A, C, I, K, M and O).

During very early stages, WT showed greater expression for PsINV448 than its transgenic lines. But later, 562 showed elevated expression and at 10 DAF, line 382 showed maximum expression in pod walls (Fig 4.8 A). Similarly, line 5156 showed greater expression for this gene family member during early stages of development, but at 7 DAF, the parental lines 1/55 showed higher expression in pod walls (Fig 4.8 C).

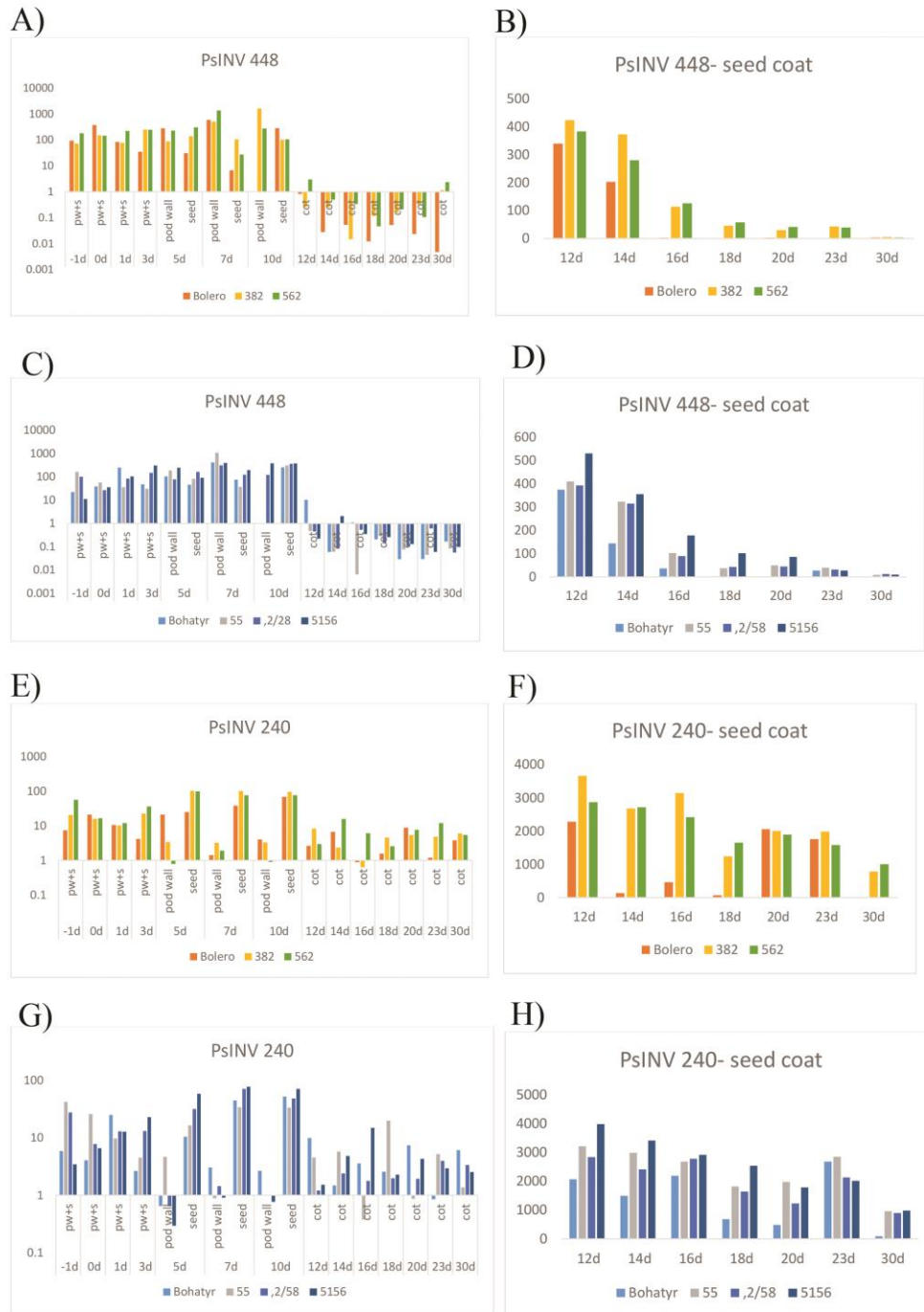
PsINV285 was expressed highly during very young stages of pod development (-1 to 3 DAF), especially in the double transgenic lines (Fig 4.8 I). Line 562 showed greater expression at most of the stages, except 3 DAF at which 382 showed the highest expression. In field pea and its transgenics, this gene family member showed up-regulation only at -1 DAF, then the expression decreased (Fig 4.8 K). During maturation stages, this gene family member was expressed very little.

PsINV320 showed almost similar expression pattern as of PsINV285. Both the double transgenic lines (382 and 562) showed increased expression for this gene family member. On the other hand, both single and double transgenic lines showed elevated expression among field peas. But it can be seen that the parental lines (55 and 2/58) showed greater expression most of the time (Fig 4.8 M, O).

PsINV240 showed a different expression pattern. This gene family member showed a continuous expression throughout the development (Fig 4.8 E, G). Also, this gene family member was highly expressed in young seeds (7 and 10 DAF), rather than in pod walls. Both the double transgenic lines (382 and 562) showed high expression, but during early stages 562 had high expression, whereas 382 showed greater expression in the seeds during 5, 7 and 10 DAF. Among field peas, all the transgenic lines showed almost same level of expression, however, 5156 showed the greatest expression in young seeds and at some stages of cotyledons (Fig 4.8 G).

PsINV240 was the most highly expressed GFM in seed coats. All the PsINV gene family members showed similar pattern of expression, that is greater expression at early stages and less expression at during the storage phase. PsINV448 was highly expressed at 12 and 14 DAF. Transgenic lines showed greater expression than WT. Among field pea lines, the double transgenic line 5156 showed the greatest expression at all stages. But at maturity, the expression was much less or negligible, both in process peas and field peas (Fig 4.8 B, D). PsINV240 was expressed in almost all stages. Both the transgenic lines showed more elevated expression than WT. Between the field pea lines, the double transgenic line 5156 showed the greatest expression in most of the developmental stages. Among the parental lines, 1/55 showed more elevated expression compared with 2/58 (Fig 4.8 F,H).

PsINV285 was the least expressed GFM in seed coats. Expression was more during early stages than later, and transgenic lines showed greater expression than WT. At maturity, line 562 showed elevated expression. Among field pea lines, 5156 showed greater expression at most of the stages (Fig 4.8 J,L). PsINV320 was more expressed during early stages than the later stages. But at maturity, all the lines had some expression. Also, transgenic lines had greater expression than WT. Between the field pea lines, the line 5156 showed the greatest expression, relative to others (Fig 4.9 N,P)



Developmental Stages





**Figure 4.8: Relative expression of PscwINV gene family members in developing seeds of WT and transgenic lines.**

## 4.4 Discussion

In this chapter, expression of genes affecting both nutrient transport and seed development were analysed in wild type and transgenic pea plants. Transgenic pea plants were previously developed by Plant & Food Research Institute, Lincoln.

Earlier studies demonstrated the importance of transporters for seed N nutrition using plants overexpressing amino acid transporters. It was found that uptake of amino acid, total seed N and protein content were increased when expressing *VfAAP1* in *Vicia* and pea under control of an embryo specific promoter (Rolletschek et al., 2005a). In addition, seed size, weight, and protein content were elevated in the transgenic plants. However, overall seed yield was not changed in the pea plants when grown in the greenhouse or in the field (Rolletschek et al., 2005a). This suggests that increased activity of sink-located transporters positively affects sink strength and N uptake rather than influencing sink numbers. A later study showed that the transgenic AAP1 seeds grown under growth chamber conditions or the field showed a significant increase in N and protein content. However, AAP1 seeds had reduced carbon content, starch and sucrose levels in addition to reduced seed size (Weigelt et al., 2008).

Recently, another study revealed that the transgenic pea plants over-expressing two *AtAAP1* genes had increased final seed yield, soluble protein content and increased N uptake even though the seed weight was not affected (Zhang et al., 2015).

Another study by Rosche et al. (2002) demonstrated that *StSUT1* under control of the pea vicilin promoter, was functionally overexpressed in storage parenchyma cells of developing pea cotyledons. Heterologous expression of *StSUT1* led to a substantial increase in the potential capacity of storage parenchyma cells to transport sucrose from the seed apoplasm. However, this study did not show any increase in the final seed yield or any detectable change in final dry weight of seed (Rosche et al., 2002), and they suggested that there are other processes other than photoassimilate import also influence final seed biomass.

### 4.4.1 Increase in yield

Phenotypic characters were analysed in both parental lines and the double transgenic lines by Plant & Food personnel. The results showed that there was a significant increase in yield in two double homozygote seed lines, which contain both the transgenes. Data also showed that

there was an increase in the seed number which includes increase in seeds per pod and pods per plant, although there was not much increase in the seed weight. The yield increase could be a result of over-expression of the transporter genes during the early stages of seed development.

#### **4.4.2 Increased expression of transporter genes**

In this experiment, transgenic plants engineered with two transporter genes, *PsSUT1* driven by *AtAAP1* promoter, and *PsAAP1[3]* driven by either the 35S promoter or *AtAAP1* were studied. As 35S is the constitutive promoter, it is expected to express all over the plant, and *AtAAP1* promoter is expected to express in se/cc complex of the leaf phloem parenchyma and to the epidermal layer of pea cotyledons (Tegeder et al., 2007).

From the results of the phylogenetic analysis, it was revealed that the *PsAAP1* (Accession code: AY956395) – a sequence previously submitted to GenBank by Tegeder *et al.* (2000) is most similar to *AAP3* in other species. However, since the *PsAAP1* sequence identified by Dr Song is most similar to *AAP1* sequences in other species, it was decided to update the nomenclature and refer to this as the ‘real’ *PsAAP1*. Hence, it is important to note that the *PsAAP1* referred to in previous publications by Tegeder *et al.* (2000 and 2007) is actually *PsAAP3*, and is shown as *PsAAP1[3]* in this study.

Expression of genes was studied for 33 target gene family members, throughout seed development. It is evident from the data that even at the earliest stages of pod and seed development, both the transgenes (*PsSUT1* and *PsAAP1[3]*) showed elevated expression. The most interesting result was that these transgenes also affected the expression of other gene family members, for example, *PsAAP2*, *PsAAP6*, *PsSUF1*, as well as other genes. This is in contrast to other transgenic work which did not show up-regulation of other gene family members. For example, the transgenic plants over-expressed *VfAAP1* did not show any effect on other genes (Rolletschek et al., 2005b). However, this result does agree with the finding from Zhang et al. (2015), which states that there was an up-regulation of sucrose transporter, *SUT1* in transgenic plants, over-expressing *PsAAP1[3]*, but not of other *AAP* gene family members.

During seed development, photoassimilates are transported to the sink tissues from source leaves. Transporter genes, especially amino acid transporters and sucrose transporters play a

crucial role in this. Sucrose transporters are co-localized to plasma membranes of sieve element/companion cell (se/cc) complexes with H<sup>+</sup>-ATPases. During the earliest stage of seed development, the seed coat development is dominant, whereas the embryo is still undifferentiated. Then the thin walled parenchyma starts to differentiate to become the unloading region of assimilates into the endospermal vacuole (Weber et al., 1996). In other words, the maternal seed coat modifies and controls the nutrient supply to the embryo during early legume seed growth, thus regulating the cell division phase (Weber et al., 1997). Nutrient transporter gene play an important role in unloading the photoassimilates in these regions.

In developing pea cotyledons, sink limitation is imposed by processes responsible for starch and storage protein accumulation (Wang and Hedley, 1991). Imported sucrose and amino acids contribute carbon and nitrogen skeletons for the biosynthesis of these storage compounds (Patrick and Offler, 2001). Sucrose, released from seed coats, is taken up from the seed apoplasm by abaxial epidermal transfer cells, subepidermal cells, and one or two outer layers of underlying storage parenchyma cells of pea cotyledons by sucrose transporters (Tegeder et al., 1999; Rosche et al., 2002).

It was shown in the data that the sucrose transporter genes are up-regulated at the similar stages. Cell division is at its maximum at the early stages and requires energy. Therefore, in this case sucrose provides the energy for cell proliferation which is why the *SUT* genes are up-regulated.

The data shows that *PsSUT1b* was highly expressed in the pod walls, whereas the sucrose facilitator (*PsSUF1*) was expressed predominantly in seeds. The transgenic lines showed greater expression than that of WT. And also, the double homozygotes had more elevated expression than the parental lines. The sucrose facilitator gene, *SUF1*, exhibited tissue specificity, as it was expressed more highly in the seed tissues than in pod walls and/or other developmental stages. This result agrees with those of Zhou *et al.* (2007), where they found that *PsSUF1* expression was strongest in cotyledons and coats of developing pea seeds (Zhou et al., 2007).

#### **4.4.3 Effect of ectopic expression of transporter genes on cytokinin regulatory genes**

The data revealed that the cytokinin regulatory genes (*IPT* and *CKX*) are expressed during the early stages during which cell division is maximal, with the exception of *PsIPT4* which was expressed more in the maturation phase. Interestingly, the expression is greater in transgenic lines which means that the transgenes (*SUT* & *AAP*) may have indirectly affected the activity of the cytokinin genes.

The relative expression differs in various tissue types and at different developmental stages (Weber et al., 1997, Miyawaki et al., 2004). That means, the tissue specificity of *PsIPT1*, *PsIPT2* and *PsIPT3* was observed in the organs. Le et al. (2012) mentioned that the transcript levels of *GmIPT* in flowers, pods and seeds were different in each tissue which meant that each of these tissues required different *GmIPT* genes for cytokinin biosynthesis and that the combined action of at least five *GmCKX* genes is required for maintaining cytokinin homeostasis in flower tissue (Le et al., 2012). O'Keefe et al. (2011) found that in *Brassica rapa*, there was an interesting pattern in the expression of *IPT* and *CKX* genes, that is., as *BrIPT* expression increased and decreased, *BrCKX* showed the similar pattern (O'Keefe et al., 2011b, Song et al., 2015) and it has also been suggested that this is a causative relationship (Brugière et al., 2008).

The expression levels of *GmCKX* genes in flowers, pods and seeds were studied by Le et al. in 2012. It was found that high expression of *GmCKX8* was in pods. The relative expression varied in seeds, pod walls and leaves as Werner et al. (2003) reported about differential expression of *AtCKXs*. Interestingly, this data reveals that there is an elevated expression of isolated *PsCKX4*, *PsCKX5* and *PsCKX2* in pod walls, which agrees with the finding from Werner et al. (2003). The *AtCKX* genes were localized to specific domains in the root and shoot (Werner et al., 2006). *PsCKX1* showed high expression in early developing seeds of process peas. At the same time, *PsCKX3* did not show any expression in the developing seeds.

According to the data, transgenic lines showed greater expression than the WT, especially the single transgenic parental lines showed elevated expression for the cytokinin biosynthetic genes. This may be due to the presence of the transgene which regulates the other genes as

well. Also, during the storage phase, the transgenic lines showed up-regulation for *PsIPT* gene family members.

#### **4.4.4 Effect of ectopic expression of transporter genes on cell wall invertase**

Expression of cell wall invertase has been observed in many systems (Roitsch and González, 2004), including developing fruit (Hayes et al., 2007) and seeds (Weber, 2005). Importantly, elevation of the endogenous *CWIN* activity significantly increased seed weight and fruit sugar hexoses in tomato and grain size in rice respectively, either by silencing its inhibitory protein (Jin et al., 2009) or over-expressing the proteins with its own promoter (Wang et al., 2008). These findings show that the development of seed and fruit is limited by *CWIN* activity in these crops. Another work showed that the mutant seed had a loss of 98% of total invertase activity in maize kernels and reduction of 70% in mature seed weight (Carlson et al., 2000).

As suggested in the literature for *B. napus* (e.g. Song et al. (2015)), the isolated *PsINV* gene family members exhibited higher expression during early stages of seed development and also in the seed coats. It was observed in several plant species that an increase in invertase activity occurs in response to hormones such as gibberellins {Liu-Lai Wu, 1993 #429} or cytokinins (Ehneß and Roitsch, 1997). It was not clear whether these effects are due to direct regulation of invertase genes by plant hormones or via stimulated cell proliferation creating new sinks for sucrose. In 1995, Weber et al., studied the molecular physiology of photosynthate unloading and partitioning during seed development of fava bean and found that, during the pre-storage phase, there were high levels of hexoses in the cotyledons and in the apoplastic endospermal space which correlated with high levels of cwINV in the seed coat.

It is evident from the data that transgenic lines showed higher expression for cell wall invertase gene family members than the WT, which may result in an increased pool of hexoses in seed coat and cotyledons, thereby improved sink strength. This can be due to the presence of the transgene which indirectly affects the regulation of this gene family. However, some of the members (*PsINV* 448, 240) did not show much difference in the expression level between transgenics and non-transgenics.

## 4.5 Summary

The data in this chapter is a comparison between transgenic and non- transgenic plants in the expression of various gene family members. It is evident from the data that the transgenes influenced the activity of other genes during seed development. Results clearly show that the transgenic plants had increased expression of other gene family members too. It is clear from the data that there was an increase in seed number in double transgenic plants, compared with WT, and also the increased seeds were filled, which could be the result of increased nutrient transport to the sink tissues during early stages of development, when cell division was at a maximum. This finding can be correlated with other works by Tan et al. (2010) and Zhang et al. (2015), in which they suggested that the increase in amino acid phloem loading led to higher pod set and increased seed number per pod in the AAP1 over-expressed plants.

The data also reveals that the major effect of the transgenes was seen in the seed coats, compared with cotyledons. This can be due to the seed coat is acting as a powerful sink and importing nutrients from the source.

## **CHAPTER 5**

### **Effect of the transgene on gene expression in source leaves**

#### **5.1 Introduction**

Partitioning and allocation of photosynthate play a crucial role in plant development and crop yield (Patrick, 1997). Photosynthesis is the main source of energy to support biological processes in plants. The primary sites of photosynthesis are leaves and to a smaller extent, green stems and pods. Other organs such as flowers, seeds and roots perform little if any, photosynthesis and are dependent on the photosynthetic organs. Plant tissues are grouped in to two classes based on their ability to produce or consume assimilates. They are source tissues and sink tissues (Turgeon, 1989). Carbohydrate exporting tissue is often referred to as ‘source tissue’ and the importing tissue as ‘sink tissue’. Source tissues are fully expanded leaves. Sink tissues can either be metabolic sinks like meristems or roots, where most of the imported assimilates are used for energy and only small amounts are stored temporarily, or storage sinks such as seeds and tubers, where the imported metabolites are accumulated as sucrose, starch or protein (Sonnewald et al., 1994).

Plant nutrient transporters play an important role in the source- sink transport. As starch and protein are the two major forms of storage, sucrose transporters and amino acid transporters play major roles in the transport mechanism. It is believed that phloem loading is the initial step in the transport pathway. Two major phloem loading mechanisms have been proposed: apoplasmic loading and symplasmic loading (Turgeon and Medville, 2004). In the case of apoplasmic loading, sucrose transporters (SUTs) have been identified as the essential factors for sucrose translocation (Lalonde et al., 2004). Amino acid transporters have been identified in various plant species and it is clear that they exist as members of gene families with transport properties (Rentsch et al., 2007).

Another membrane protein family which has recently been identified as assisting in the transport of photoassimilate from source to sink is SWEET. SWEETs are a newly identified class of sugar transporters, which have been identified as a prerequisite for SUT- mediated



phloem loading (Chen et al., 2012a) that facilitate diffusion of sugars across cell membranes down a concentration gradient.

In this experiment, two different kinds of source leaves were analysed for their gene expression to study the source- sink interaction and the impact of source leaves on final seed yield, both in transgenic and non- transgenic plants.

## **5.2 Materials and methods**

The materials and methods used for this experiment are explained in chapter 2. The samples used in this experiment were developing leaves and mature leaves. The leaf samples were collected at time of sampling of pods. Different developmental stages of leaves were collected as samples. Leaves supporting the pods of 5, 10, 15, 20, 25 DAF and senescence leaves were collected.

For this analysis, leaves of two different developmental stages, that is, 5 and 20d were chosen. Both types of leaves were fully expanded but those associated with pods 5 DAF are here referred to as “young” and those collected when pods were 20 DAF are referred to as “older” source leaves.

## **5.3 Results**

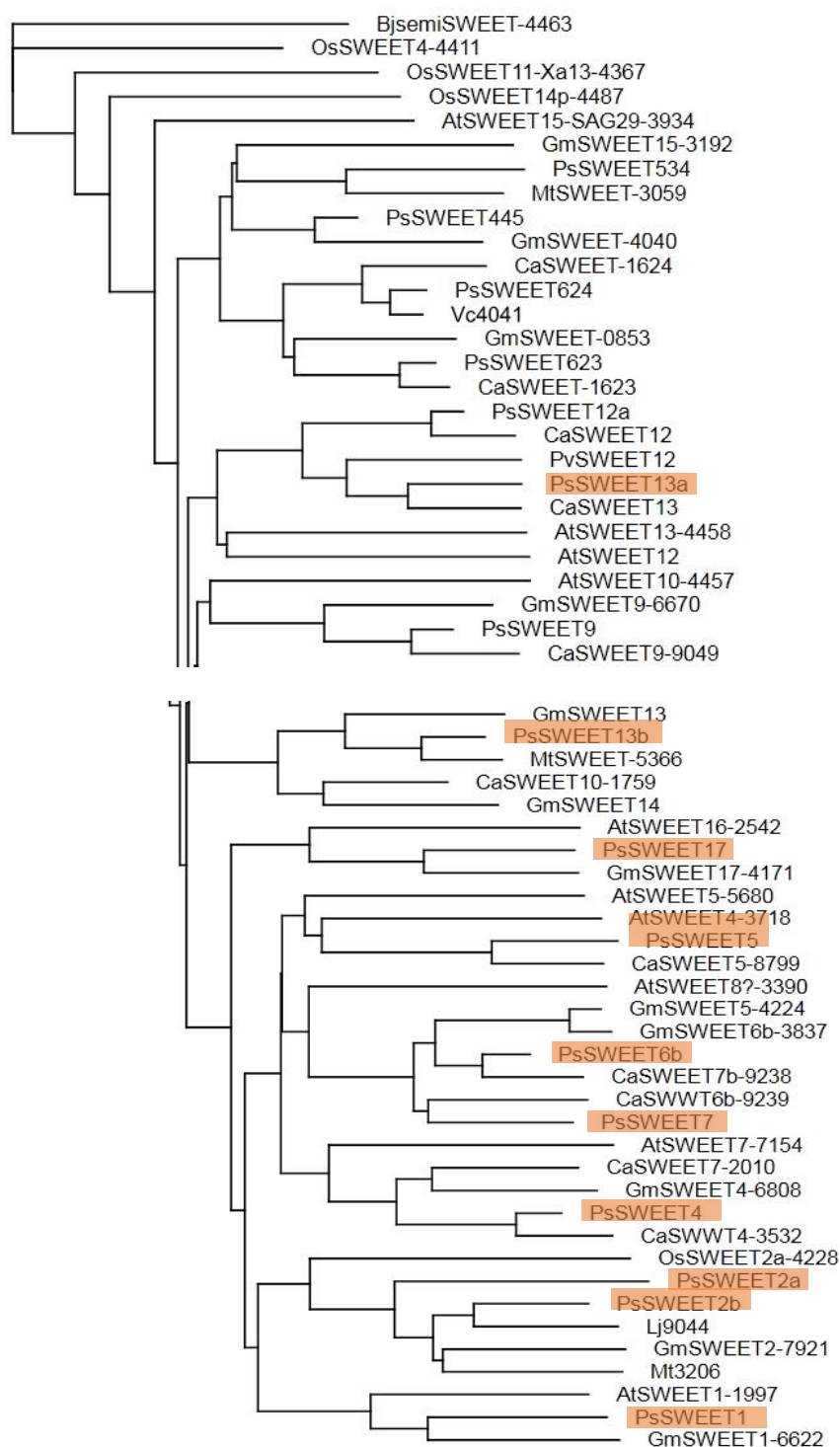
### **5.3.1 Identification and confirmation of *PsSW* gene primers**

SWEET (*PsSW*) gene family members were identified using a BLAST search of the GenBank database. Pea transcriptome sequence data were used by Dr Jason Song to design the primers. Based on the result from temperature gradient PCR cycling, the standard PCR program to amplify the DNA fragments was carried out. Primer sequencing and DNA purification was done as explained in Chapter 2 (Section 2.2.6). Thirty-six pairs of primers were designed for different SWEET (*PsSW*) gene family members. The products of the primers highlighted in Table 2.6 were sequenced. The sequence verification after sequencing was done for each specific target gene with BLAST (<http://www.ncbi.nlm.gov/BLAST>) searching the GenBank database and each confirmed target gene was selected and identified as *PsSW* gene family members.

### 5.3.2 Phylogenetic analysis of *PsSW* gene

SWEET primers were identified by Dr Jason Song, by using the pea transcriptome. The phylogenetic analysis was done by including the sequences of *A. thaliana*, *P. sativum* and other legumes including *M. truncatula*, *G. max*, *L. japonica* and *P. vulgaris* for SWEET genes along with the isolated *PsSW* gene families by MEGA4 program. The tree was rooted with *Bradyrhizobium japonicum* SemiSWEET (*BjsemiSWEET*) and *OsSWEET* as out groups.

When the phylogeny was completed, the tree showed seven main clades. The isolated *PsSW2* grouped with *GmSW2*. The isolated *PsSW6b* was grouped with *CaSW7b* and *GmSW6b*. *PsSW17* formed a clade with *GmSW17* and *AtSW16*. *PsSW13b* formed a clade with *GmSW13*.



**Figure 5.1: Phylogenetic tree of SWEET.** The *PsSW* gene family members isolated and used in this study are highlighted. The tree was rooted with *Bradyrhizobium japonicum SemiSWEET* (*BjsemiSWEET*).

### 5.3.3 Gene expression analysis

All the melting and amplification curves of different gene family members (GFM) are shown in the appendix Section 5.2.

#### 5.3.3.1 Expression analysis of *PsSW* gene in leaves supporting pods of different ages

The relative expression of *PsSWEET* gene was studied in leaves supporting developing pods of different ages of parental lines and transgenic seed lines. Out of fifteen isolated gene family members, eleven members were used to study gene expression in the leaves.

*PsSW1*, 2a, 2b, 13a, 13b, 17 gene family members showed strong expression in leaves, with greater expression in younger than in older source leaves. The transgenic lines showed greater expression than their parental wild type for most of gene family members identified.

The isolated *PsSW1* showed greater expression in the young source leaves compared with older source leaves of both transgenic and non-transgenic. Among the transgenic lines this gene family member was more highly expressed in field pea varieties (Fig 5.2 A).

*PsSW2a* had a low expression in the older leaves of all seed lines, whereas it showed elevated expression in young source leaves. Three of the four double transgenics had more elevated expression than the respective parental line (Fig 5.2 B).

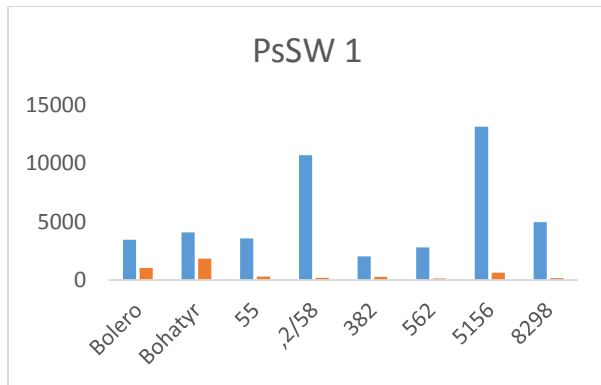
*PsSW2b* showed similar level of expression for the transgenic except 8298 line which showed very high expression in young leaves. Wild type showed much less expression in young leaves (Fig 5.2 C). *PsSW13a* and 13b showed similar expression patterns, that is higher expression in transgenic than the wild type (Fig 5.2 I, J). However, 13b was particularly strongly expressed. In contrast, *PsSW17* had greater expression in young leaves of wild type than the transgenic lines (Fig 5.2 K).

*PsSW4* had similar expression pattern as *PsSW1* but this gene family member showed comparatively less expression (Fig 5.2 D).

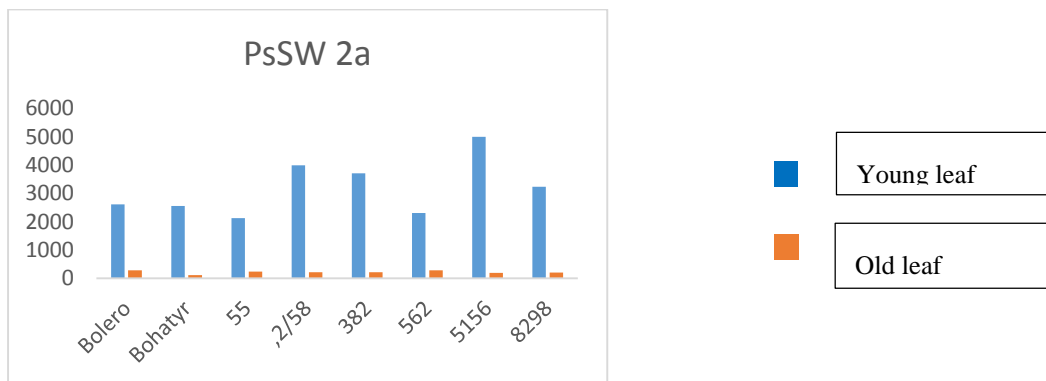
Relative to *PsSW1*, 2a, 2b, 13a, 13b and 17 gene family members, *PsSW5*, 6a, 6b and 7 showed negligible expression both in young and old source leaves of all the seed lines

although in 6b expression was more elevated in the older leaves of WT (Fig 5.2 E, F, G and H).

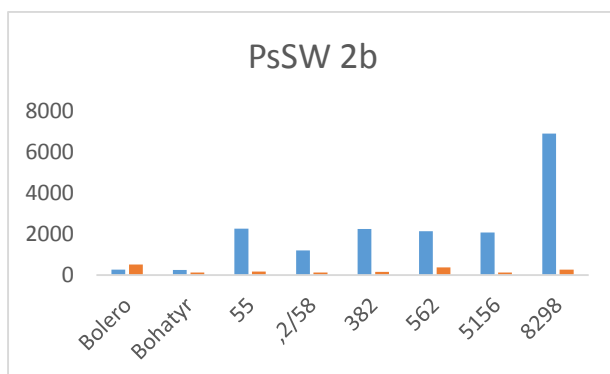
A)



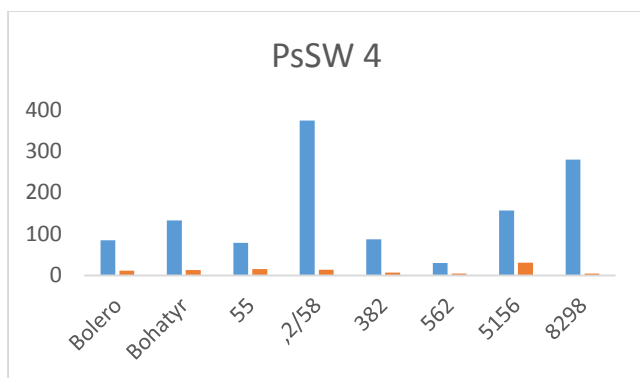
B)



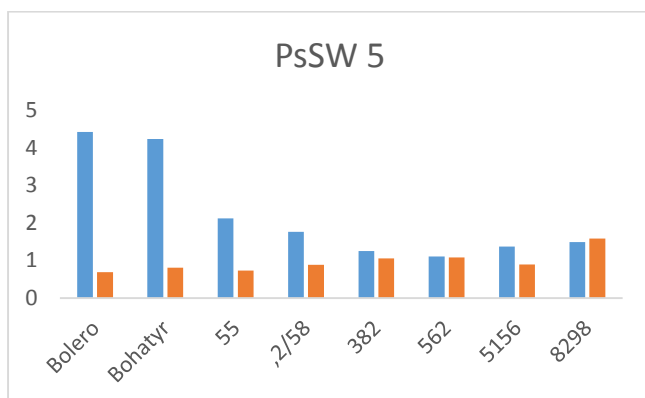
C)



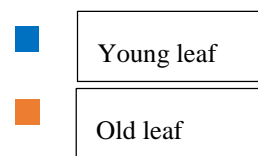
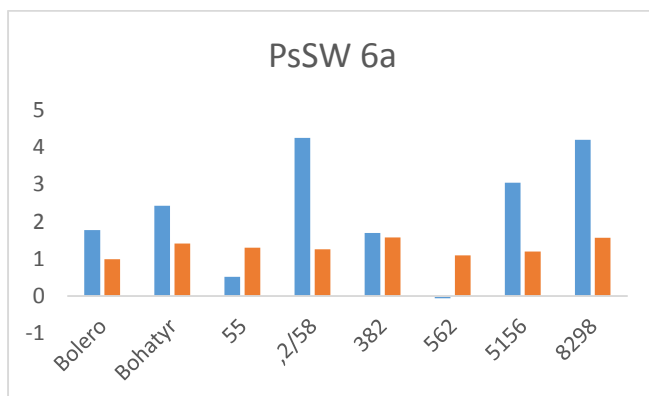
D)



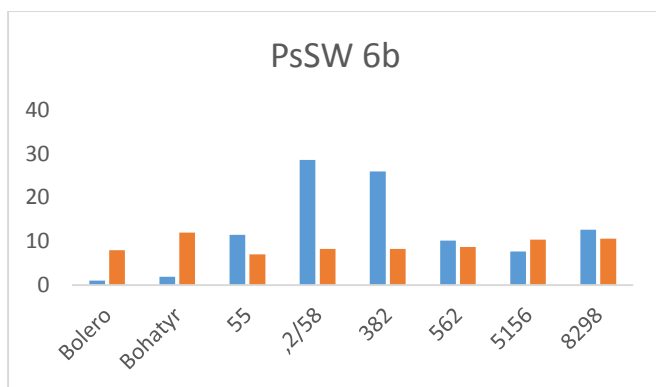
**E)**



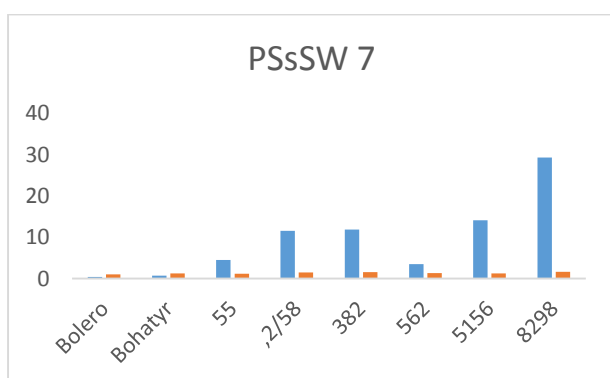
**F)**



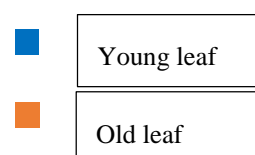
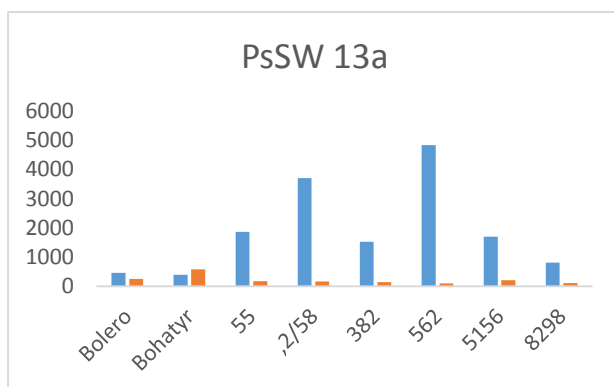
**G)**



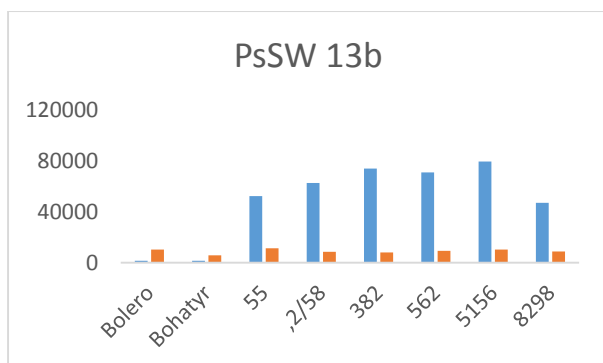
**H)**



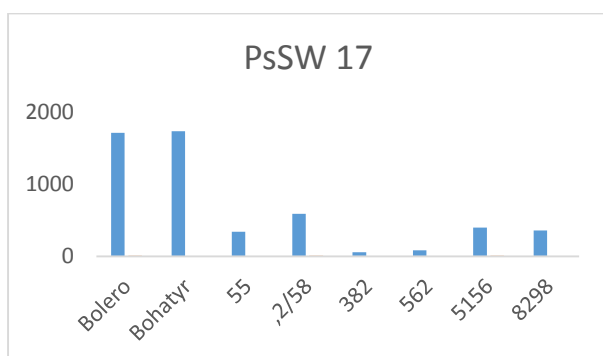
**I)**



**J)**



**K)**



**Figure 5.2: Relative expression of PsSW gene family members in different stages of leaves of WT and transgenic lines.** WT- Bolero and Bohatyr, parental lines- 55 and 2/58, double transgenic lines- 382, 562, 5156 and 8298.

### ***5.3.3.2 Expression analysis of PsSUT gene in leaves supporting pods of different ages***

All the five isolated *PsSUT* gene family members were used to study the gene expression in young and old source leaves. Among these five, *PsSUT2* showed negligible expression in leaves. All others had good expression in leaves.

In this study, *PsSUT1a* primers should detect the *AtAAP1::PsSUT1* transgene. The up-regulation of this gene family member in the transgenic lines showed that the transgene was detected. *PsSUT1a* is the greatly expressed gene family member in leaves. Also, this gene family member was highly expressed in young leaves of double transgenic lines, 382 and 8298. But, in WT this gene family member was expressed greater in old than young leaves (Fig 5.3 A). *PsSUT1b* showed higher expression in the old leaves relative to young leaves of

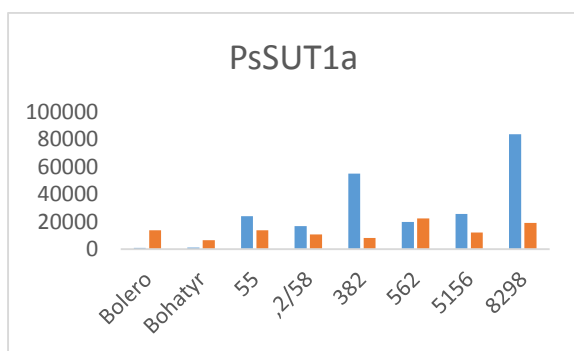


wild type. In the young leaves, this gene family member showed higher expression in the transgenic lines (Fig 5.3 B).

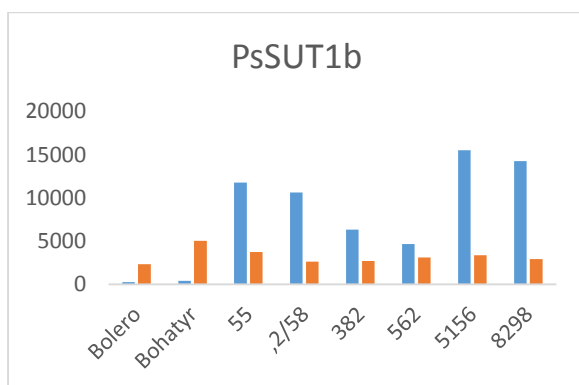
*PsSUT2* showed greater expression in old leaves than young leaves in all seed lines. But the expression was much lower in leaves when compared with other gene family members (Fig 5.3 C, note the scale). *PsSUT10* showed greater expressions in young leaves of WT and in field pea transgenic lines, but not in all the process pea transgenic lines (Fig 5.3 D).

*PsSUF1* showed similar pattern of expression in leaves as *PsSUT10*. The process pea varieties hardly showed any expression for this gene family member in young leaves, but slightly more expressed in the source leaves (Fig 5.3 E).

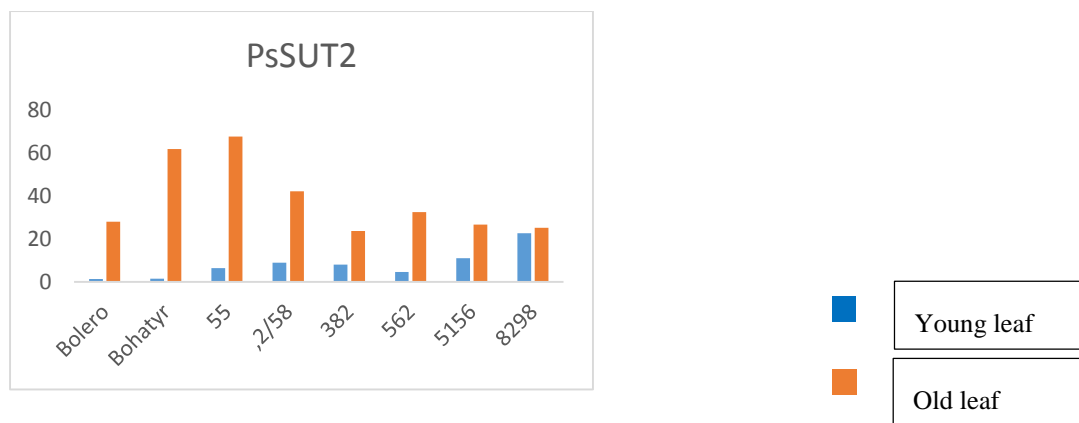
A)



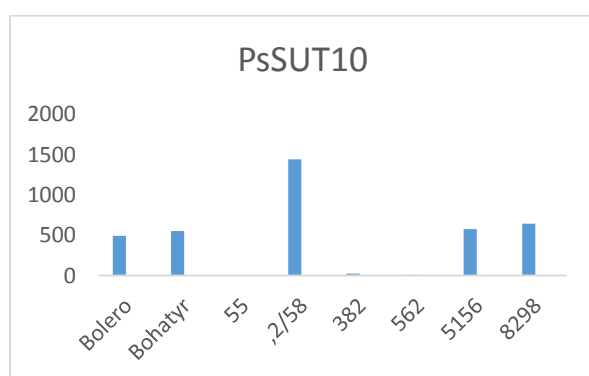
B)



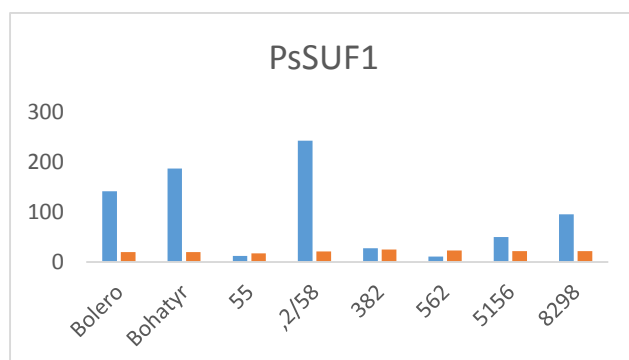
C)



**D)**



**E)**



**Figure 5.3: Relative expression of PsSUT gene family members in different stages of leaves of WT and transgenic lines.**

### ***5.3.3.3 Expression analysis of PsAAP gene in leaves supporting pods of different ages***

The *PsAAP* transgene was detected by the *PsAAP3a* primer. Elevated expression was detected for this gene family member. The transgenic lines showed greater expression of *PsAAP3a* than the WT both in young and old leaves. Between the process pea lines, 382 and 562 showed greater expression in their older source leaves. Field pea lines did not show much difference between the young and old source leaves in expression (Fig 5.4 A).

WT showed differential expression pattern for different gene family members. They had greater expression in old leaves for GFM *PsAAP3b*, *2a*, *2b*, *2c*, *2d*, *2e*, *7c* and *7e*, compared with young leaves, whereas *PsAAP1* and *6c* showed more elevated expression in young leaves although *6c* had relatively low expression level. *PsAAP6d* did not show much difference in expression between young and old leaves in WT.

*PsAAP1* showed greater expression in the young leaves of field peas and its transgenics, and also in the line 8298, which is a process pea transgenic line. There was no difference in the old leaves in WT or transgenics (Fig 5.4 B).

Among the *PsAAP2* gene family, *2a* was the most highly expressed GFM both in young and old leaves. In all the transgenic lines, this GFM was greatly expressed in young leaves, compared with old leaves and WT. GFM *2b* was similarly increased in the young leaves relative to WT. *2c* mimicked the same pattern of expression (Fig 5.4 C, D, and E).

For *2d*, all the transgenic lines showed more elevated expression in young leaves, compared with WT young leaves, in contrast to WT which had greater expression in source leaves. *2e* was more expressed in the young leaves of most of the transgenic lines, except the parental line 1/55 and double transgenic line 562 (Fig 5.4 F and G), again in contrast to WT.

*PsAAP3b* was highly expressed in the young source leaves of all transgenic lines compared with WT. But in WT, this GFM was more expressed in old leaves compared with young leaves. Field pea lines showed more expression than the process pea lines (Fig 5.4 H).

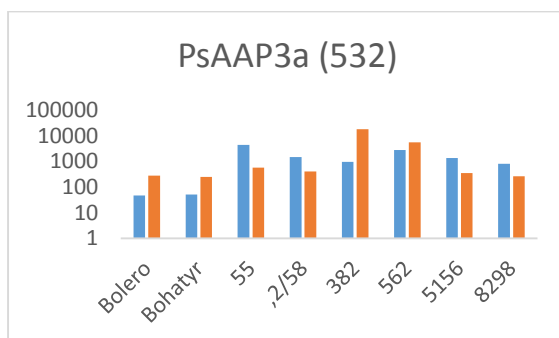
*PsAAP6c* and *6d* showed the similar pattern of expression. Both the members showed greater expression in the sink leaves of field peas, whereas process pea lines showed much less

expression in their sink leaves. Both varieties showed negligible expression in their source leaves (Fig 5.4 I and J).

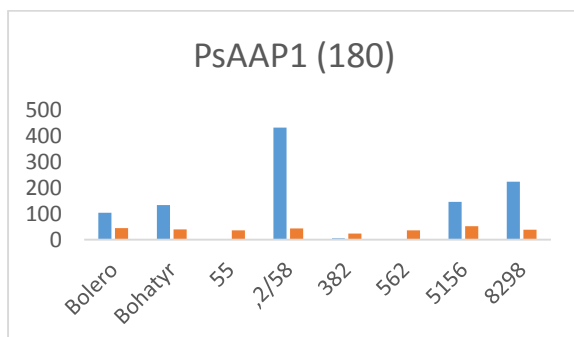
Among the *PsAAP7* gene family, *7b*, *7c* and *7d* showed somewhat similar expression pattern in all the lines. *7b* was more expressed in the source leaves of process pea lines (both WT and transgenic lines). But in field peas, the greater expression was shown in the sink leaves. *7c* and *7d* were expressed both in source leaves and sink leaves of both the varieties (Fig 5.4 K, L and M).

The *PsAAP7e* primer detected increased expression in the young leaves of all transgenic lines compared to their WT, where in contrast, the old WT leaves showed greater expression compared with young WT leaves (Fig 5.4 N).

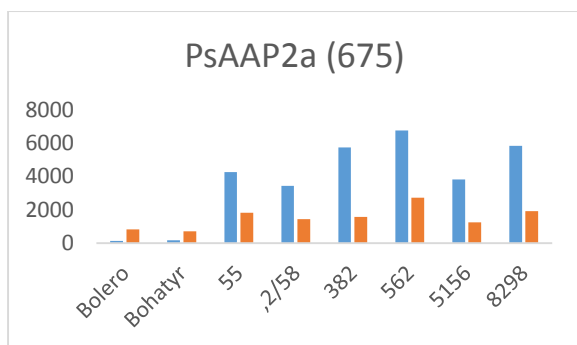
**A)**



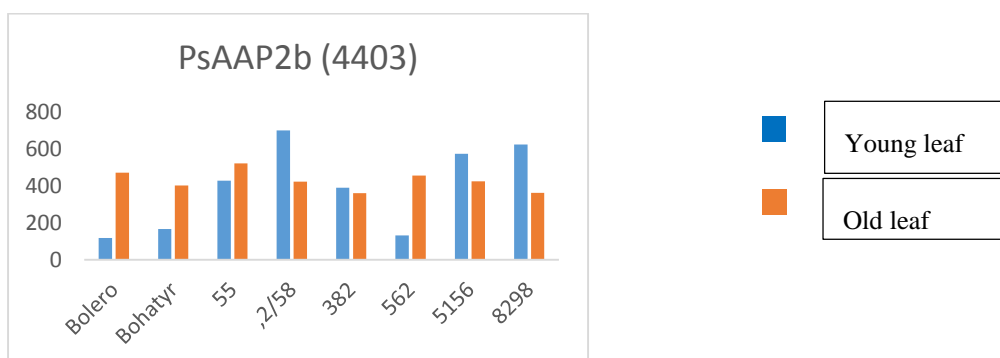
**B)**



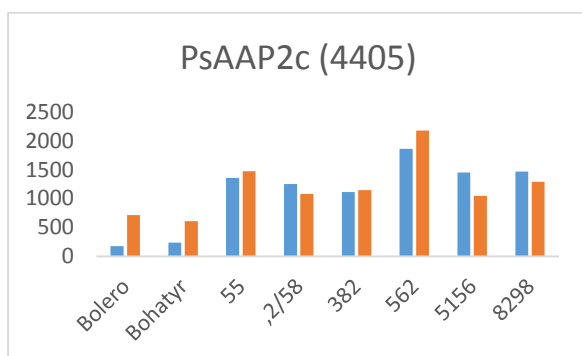
**C)**



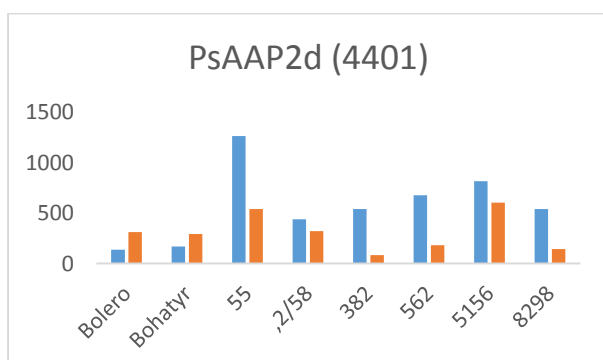
**D)**



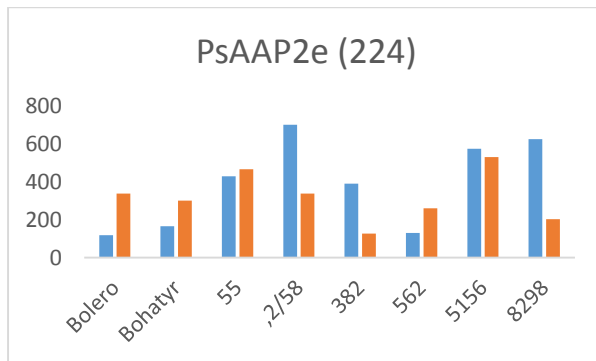
**E)**



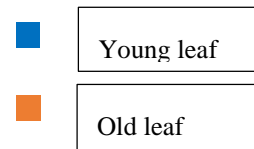
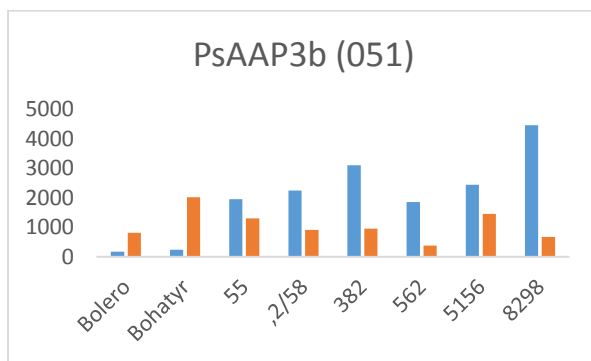
**F)**



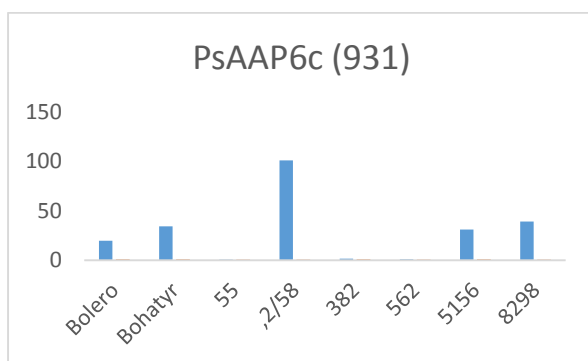
**G)**



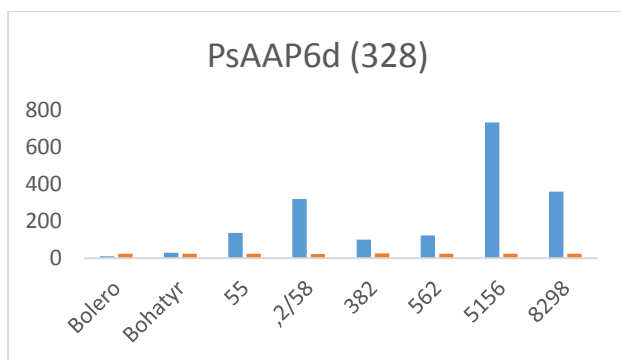
**H)**



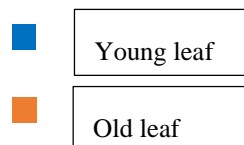
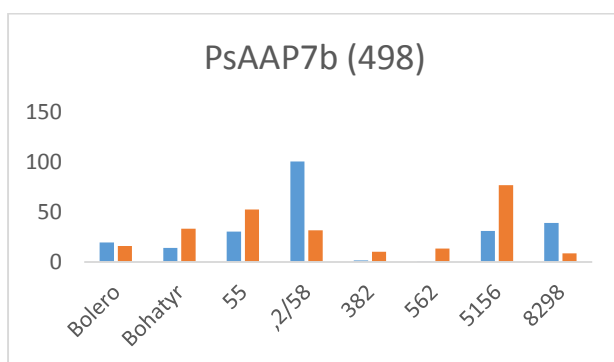
**I)**



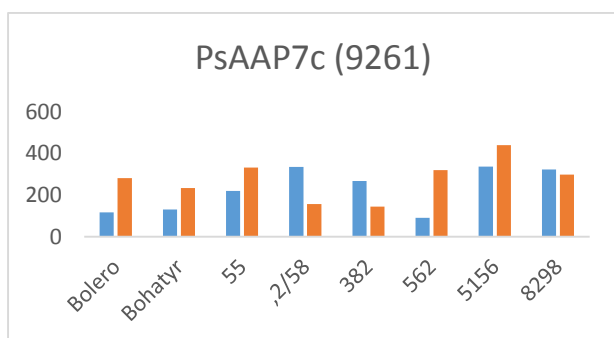
**J)**



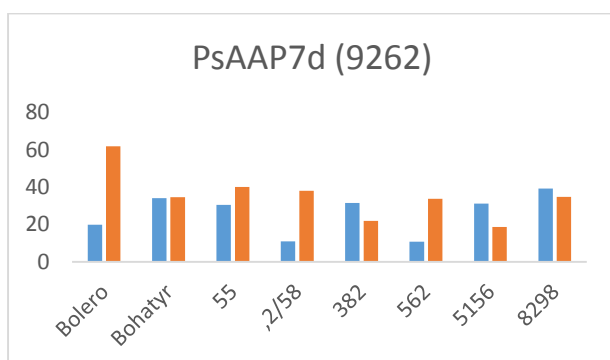
**K)**



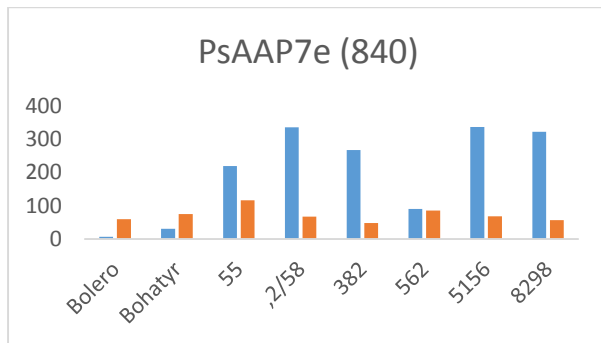
**L)**



**M)**



N)



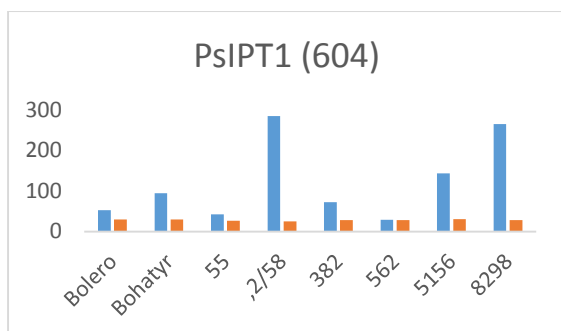
**Figure 5.4: Relative expression of PsAAP gene family members in different stages of leaves of WT and transgenic lines.**

#### ***5.3.3.4 Expression analysis of PsIPT gene in leaves supporting pods of different ages***

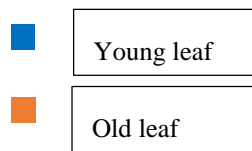
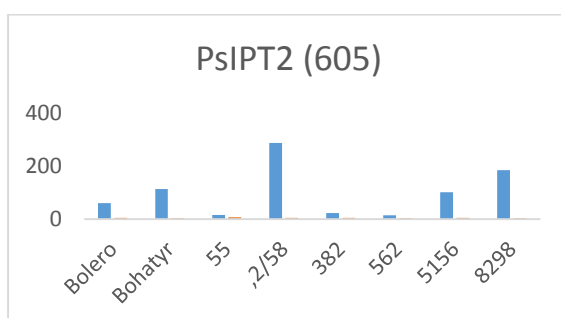
All the three isolated *PsIPT* gene family member were used to study the gene expression, all these three gene family member showed similar expression pattern in leaves. All were more highly expressed in young source leaves, and all the three showed greater expression in some transgenic lines compared with wild type. Also, among the transgenic lines, the process pea varieties were down regulated, except the line 8298 (Fig 5.5 A, B, C). Elevated expression for *PsIPT1* was shown in parental line, 2/58 which was shown through its double transgenic lines 5156 and 8298 (Fig 5.5A).

A)

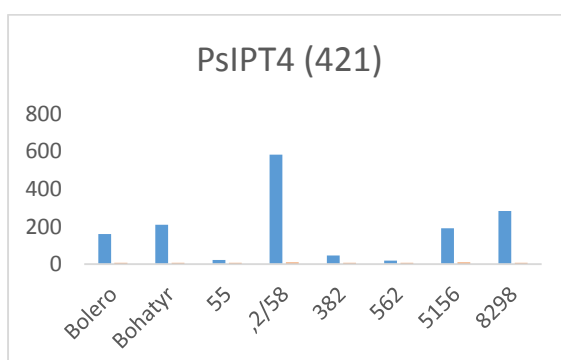




**B)**



**C)**



**Figure 5.5: Relative expression of PsIPT gene family members in different stages of leaves of WT and transgenic lines.**

### 5.3.3.5 Expression analysis of *PsCKX* gene in leaves supporting pods of different ages

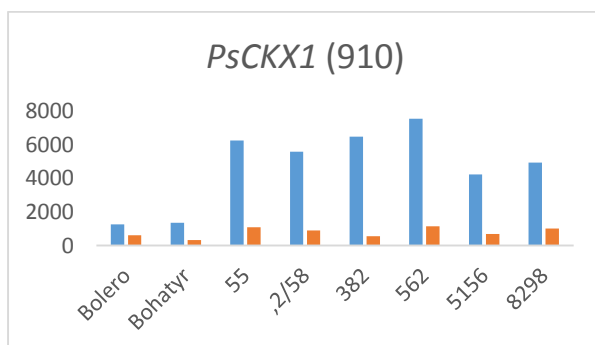
Among the six isolated *PsCKX* gene family member, three showed relatively strong expression in leaves and other three showed comparatively weaker expression.

*PsCKX1* had greater expression in young source leaves of transgenic lines relative to WT. But in the case of older source leaves, all the seed lines showed similar level of expression (Fig 5.6 A). *PsCKX4* showed comparatively less expression in leaves. This gene family member also had higher expression in younger than older source leaves, but the line 5156 showed elevated expression in older source leaves (Fig 5.6 B).

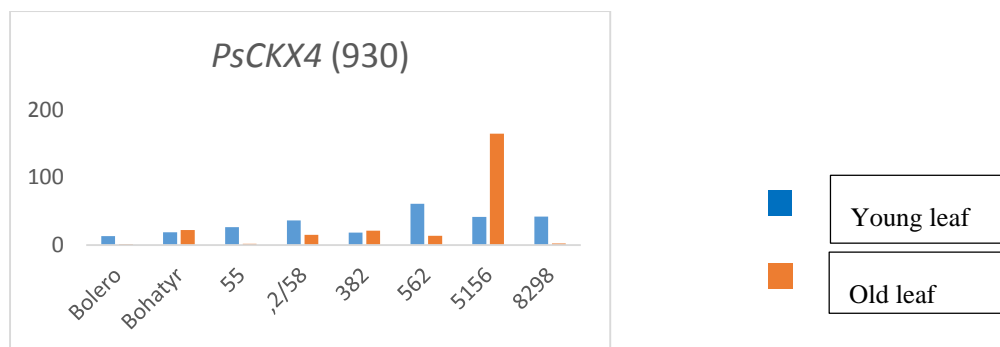
*PsCKX2* and 5 showed similar expression pattern in leaves. Both the gene family members showed greater expression in young source leaves, and several of the transgenic lines showed greater expression than the wild type in young source leaves. The elevated expression of 2/58 may be reflected of that in 5156 and 8298 (Fig 5.6 C and D). Similarly for *PsCKX6* (not sequenced).

*PsCKX3b* showed different pattern in the expression in leaves. This gene showed greater expression in the older source leaves compared to younger leaves in process peas, and in the double transgenics, but similar expression in young source leaves of Bohatyr and line 55 (Fig 5.6 F).

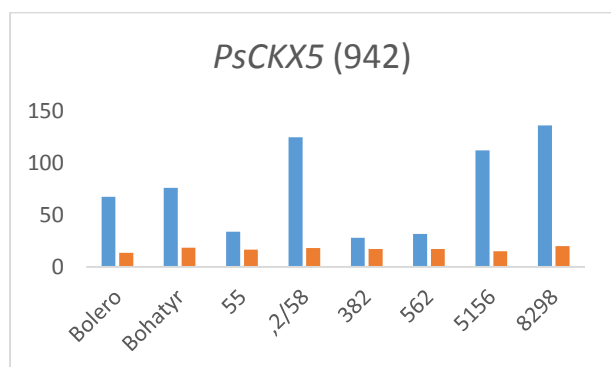
A)



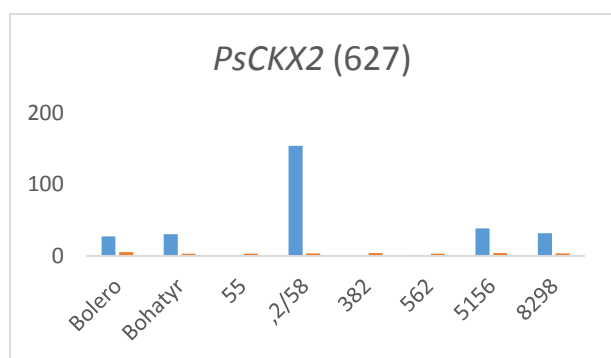
B)



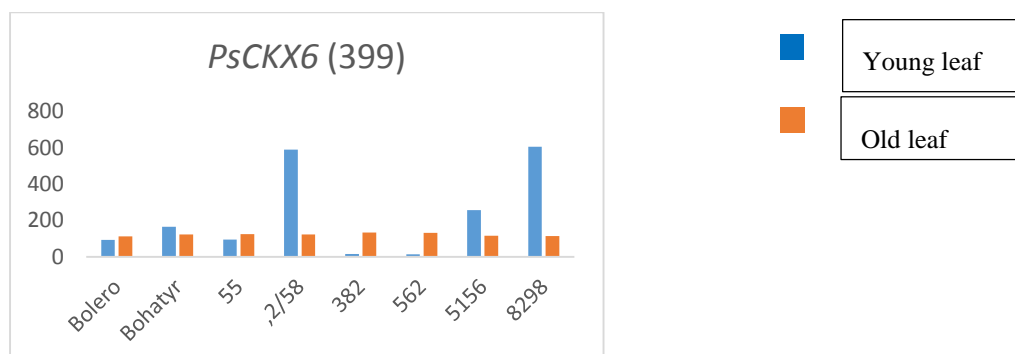
**C)**



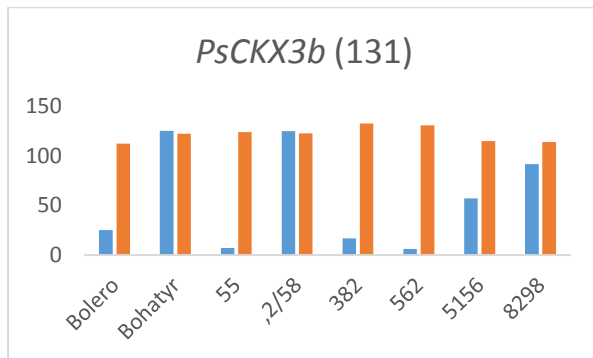
**D)**



**E)**



**F)**



**Figure 5.6: Relative expression of PsCKX gene family members in different stages of leaves of WT and transgenic lines.**

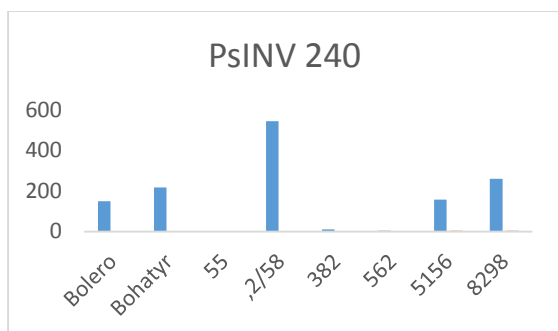
### ***5.3.3.6 Expression analysis of PsINV gene in leaves supporting pods of different ages***

Out of the 6 isolated *PsINV* gene family member, five showed strong expression in leaves, whereas *PsINV681* showed much reduced expression.

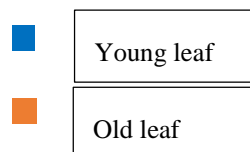
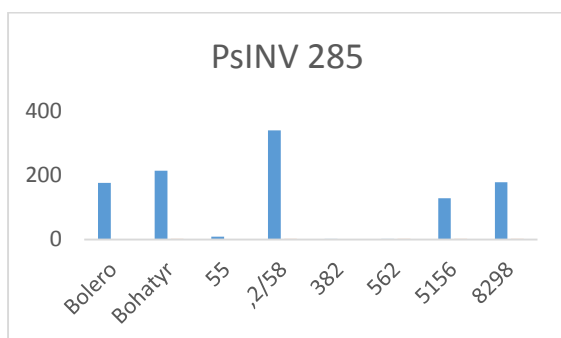
*PsINV240*, 285 and 415 showed similar expression patterns in leaves. These GFM showed elevated expression in young leaves compared to old leaves. All gene family member had slightly greater expression in young source leaves of field pea compared with process peas. Only transgenic line 2/58 showed increased expression compared with WT (Fig 5.7 A, B and D), whereas *PsINV320* showed greater expression in young source leaves of all transgenic lines especially the double transgenic lines, 382 and 5156. There was low expression of this GFM in both young and old leaves of WT (Fig 5.7 C).

*PsINV448* showed greater expression in young source than older leaves. Expression was higher in transgenic lines than wild type (Fig 5.7 E). In contrast, *PsINV681* showed reduced activity in the young leaves of all transgenic lines, relative to WT. There was little activity of this GFM detected in older source leaves (Fig 5.7 F).

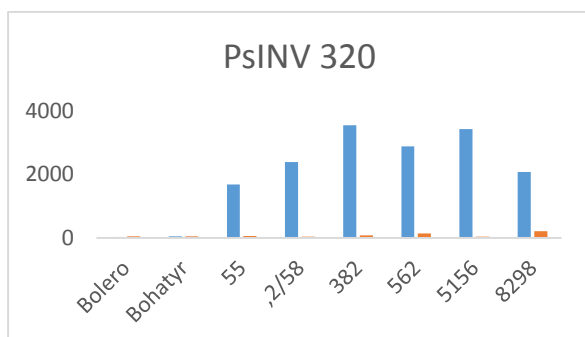
**A)**



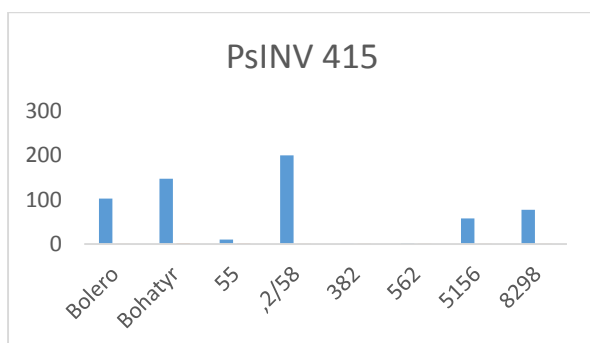
**B)**



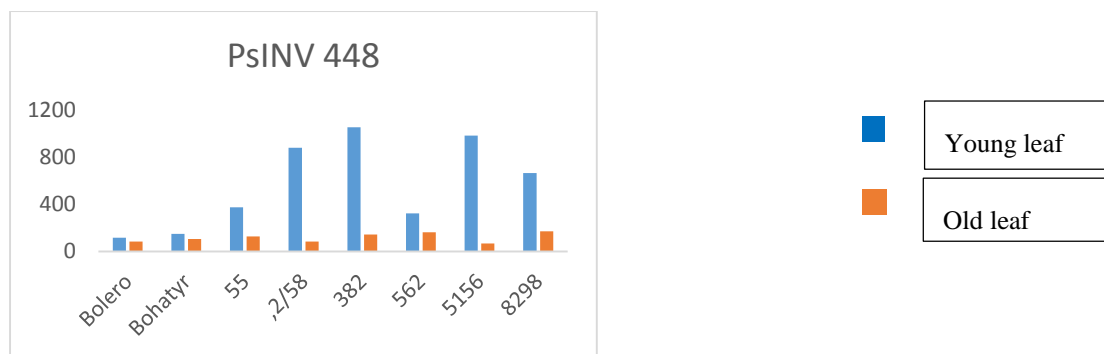
**C)**



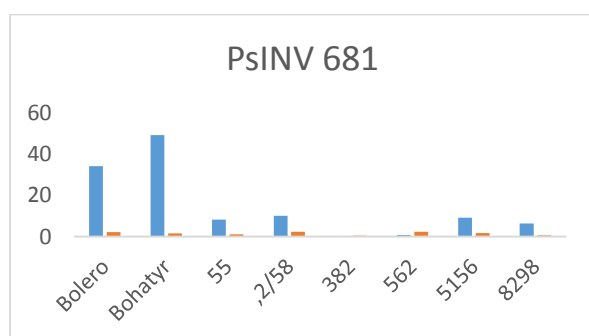
**D)**



**E)**



**F)**



**Figure 5.7: Relative expression of PsINV gene family members in different stages of leaves of WT and transgenic lines.**

## 5.4 Discussion

In this chapter, expression of different gene family members was studied in two developmental stages of leaves. The main focus was on the effect of the transgenes on the expression pattern of target genes in young and old source leaves. The transgenes used in this experiment were driven by either the 35S promoter or AtAAP1. As 35S is the constitutive promoter, it is expected to express all over the plant, and AtAAP1 promoter is expected to express in se/cc complex of the leaf phloem parenchyma and to the epidermal layer of pea cotyledons (Tegeder et al., 2007).

### 5.4.1 Identification, characterization and expression of *SWEET* gene

In this experiment, multi gene family members of coding for the main phloem loaders, *SWEET* was isolated from *P. sativum*. The relative expression of the various gene family members was studied to understand the spatial and temporal expression pattern of these

genes. The multi-gene family members were initially isolated following the analysis by Dr Jason Song of a pea transcriptome. Some genes that were detected in the pea transcriptome were not selected for the final expression studies since their expression was very low or undetectable following RT-qPCR analysis.

When the phylogeny was completed, the tree showed seven main clades. Phylogenetic analysis reveals that SWEETs are prevalent in plants and broadly conserved in eukaryotes. In eukaryotes, SWEET proteins are typically predicted to have seven transmembrane domains (Chen et al., 2010, Yuan and Wang, 2013). There are 17 *SWEET* members in *Arabidopsis* and 21 in rice (*Oryza sativa*), falling into four clades (Chen et al., 2010). More recently, using similar assays, members of clade III were found to preferentially transport sucrose across the plasma membrane. Some *SWEET*s were characterized as bidirectional, pH-independent and low-affinity sucrose transporters (Brian, 2011, Chen et al., 2012b). The close paralogs *AtSWEET11* and *AtSWEET12* of seven members in clade III share 88% similarity and are probably expressed in leaf phloem parenchyma cells, which are adjacent to the se/cc complex where phloem loading occurs. Thus, their involvement in the process of apoplastic phloem loading is strongly indicated as studies have shown the key role of *AtSWEET11* and *AtSWEET12* in phloem loading (Chen et al., 2012b).

*AtSWEET11* and *12* are highly expressed in leaves and were found to be co-expressed with genes involved in sucrose biosynthesis and phloem loading (e.g., sucrose phosphate synthase, *SUC2*). It was shown in studies that *AtSWEET10*, *13*, *14* and *15* function as sucrose transporters and, also *AtSWEET13* was expressed at low levels in leaves (Chen et al., 2012b). Another study revealed that *AtSWEET17* was expressed in the parenchyma and the vascular tissue, and functioned as a vacuolar fructose transporter, exporting fructose out of vacuoles and controlling fructose content in leaves (Chardon et al., 2013).

#### **5.4.2 Expression of *PsSWEET* gene in different stages of leaves**

In this experiment, eleven gene family members of *PsSWEET* were analysed in two different stages of leaves in WT pea plants and transgenic lines. Among the eleven GFM, *PsSWEET13a* was the most highly expressed GFM. It is clear from the data that the transgenic plants showed greater expression for *PsSWEET* gene family members, compared with WT plants. It is evident from literature that *AtSWEET11* and *12* are highly expressed in

leaves (Chen et al., 2012a) which supports the result, i.e., high expression of *PsSWEET13a* in young source leaves. Phylogenetic analysis shows that *PsSWEET13a* shows sequence similarity with *AtSWEET12* and *AtSWEET13*. The results show that the expression of *PsSWEET5* was much less in the leaves, and it can be supported by the finding that *AtSWEET5* was specifically expressed in the vegetative cells of pollen grains, indicating a role in supplying sugar for generative cell development (Chardon et al., 2013). It was revealed that mutants of *AtSWEET17* show stunted growth and lower seed yield, which indicates a role in carbohydrate allocation in plants (Chardon et al., 2013). Interestingly, the data shows that *PsSWEET17* was greatly expressed in young source leaves.

#### **5.4.3 Expression of *PsSUT* gene in different stages of leaves**

The results from this experiment show that *PsSUT1a* and *PsSUT1b* was greatly expressed in the leaves which agrees with the findings that *PsSUT1* was expressed in non-seed tissues, which suggest wider role of this gene in sucrose transport and phloem loading in leaves (Tegeder et al., 1999). Findings showed that the plasma membrane located SUTs are essential for phloem loading and normal pollen function (Sivitz et al., 2008), and all the isolated *PsSUT* genes are plasma membrane located (Fig 1.5). Evidences suggested that expression of sucrose transporters involved in phloem loading was shown to be developmentally regulated in leaves with young sink leaves showing no expression of SUT genes, whereas strong expression is seen in vascular bundles of source leaves (Wright et al., 2003). During this transition from sink to source, expression of SUT genes is initiated at the tip and proceeds towards the base of the developing leaf (Wright et al., 2003). Another study showed that the sucrose carrier from potato, *StSUT1*, was highly expressed in the phloem of the leaf minor veins, the major site of phloem loading (Riesmeier, 1993).

The result shows that the transgenic plants over-expressing *SUT1* gene shows greater expression for the transgene *PsSUT1a*, in young source leaves which suggests increased sucrose export from leaves. Another interesting finding from the result is that the presence of transgene regulates the activity of other gene family members too, *PsSUT1b* showed increased expression in young source leaves of the transgenics.



#### **5.4.4 Expression of *PsAAP* gene in different stages of leaves**

Expression of all the isolated *PsAAP* gene family members was studied in this experiment. It is seen from the data that WT plants showed more elevated expression in older source leaves for most of the GFM. In the case of WT plants, older source leaves might be uploading amino acids into the sinks. The results also show that the gene family members were up-regulated in younger source leaves of transgenic lines, which means in the double transgenic lines the transgenes are highly expressed in the young source leaves those are photosynthetically active, and the amino acids are exported to the developing sinks.

It is also clear from the data that *PsAAP3a* primer detected the transgene *PsAAP1*, driven by 35S promoter, which is why this GFM showed the greatest expression. It is also found from the result that other gene family members were also affected by the transgene. But the members of *PsAAP7* gene family members did not show great expression for leaves. Activity of AAP gene family members has been shown variously in leaves, sink tissues and roots (Tegeder, 2014). Literature showed that *AtAAP4* was associated with leaf phloem loading and *AtAAP2* was associated with phloem loading along with transport pathway (Tegeder, 2012). It is shown in the phylogenetic analysis that *AtAAP4* and *AtAAP2* have sequence similarity with *PsAAP2* gene family members. This finding agrees with the results i.e. *PsAAP2* gene family members showed greater expression in leaves.

#### **5.4.5 Expression of *PsIPT* and *PsCKX* genes in different stages of leaves**

As shown in the result, cytokinin biosynthesis genes were not highly expressed in leaves, which means cytokinin biosynthesis is very low in leaves. Evidences show that there is a very limited synthesis of cytokinin occurs in leaves (Miyawaki et al., 2004). But the results show that there is more expression in the young source leaves, compared with older source leaves. But expression of these genes were greater in the double transgenics, especially in field pea lines, compared with the WT. However, strong expression of *PsCKX* genes, especially *PsCKX1* could be seen in the result. This result agrees with the finding that cytokinin is supplied to leaves from roots, in which they suggested that roots were the major sites of tZ production and that cytokinin was transported to shoot (Hirose et al., 2008, Takei et al., 2004). Also, it was found that *OsCKX2* was expressed in leaves, inflorescence meristems and flowers (Ashikari et al., 2005b). The CKX gene family members have been shown to express

in different plant tissues and to play essential roles in controlling cytokinin levels during plant growth and development (Schmülling et al., 2003).

#### **5.4.5 Expression of *PscwINV* genes in different stages of leaves**

Most of the gene family members of cell wall invertase were expressed in the young leaves, compared with the matured source leaves. Evidences show that cell wall invertase genes are expressed in a development and organ-specific manner (Tymowska-Lalanne and Kreis, 1998). The results from this experiment shows that PsINV320 was greatly expressed in leaves, which agrees with the finding that *AtcwINV1* has been reported to be expressed in stems, leaves and roots (Tymowska-Lalanne and Kreis, 1998).

### **5.5 Summary**

In this chapter, two different stages of leaves were studied, one was young and active source leaf and the other was old source leaf which was starting to senesce.

Most of the gene family members were greatly expressed in young source leaves. The nutrient transporter genes and SWEET gene family members showed great expression in the young leaves which agrees with the suggestions from previous literatures that the nutrient transporter genes are active in the source leaves to export photoassimilate to developing sink organs such as seeds.

It is clear from the data that there is a significant increase in the source activity of the younger leaves in transgenic lines, compared with the WT. The influence of transgenes could be seen in the expression of other gene family members. It was seen in the data that young source leaves of double transgenic lines showed up- regulation of SWEET gene family members and transporter genes.

## CHAPTER 6

### Final discussion and conclusion

Until recently, plants ectopically expressing either an AAP or a SUT gene had not shown increased yield (Rolletschek et al., 2005a). The importance of transporters for seed N nutrition was previously demonstrated in studies using plants overexpressing amino acid transporters. When expressing *VfAAP1* in *Vicia narbonensis* and pea under control of an embryo-specific promoter, Rolletschek et al. (2005a) found that uptake of amino acids was increased. In addition, seed size, weight, and protein content were elevated in the transgenic plants. However, overall seed yield was not changed in the pea plants when grown in the greenhouse or in the field (Rolletschek et al., 2005b). This suggests that increased activity of sink-located N transporters positively affects sink strength and N uptake rather than influencing sink numbers. A subsequent study showed that the transgenic AAP1 seeds showed a significant increase in N and protein content. However, AAP1 seeds had reduced carbon content, starch and sucrose levels in addition with reduced seed size (Weigelt et al., 2008). Hence, they revealed that legume seeds have a high capacity to regulate and adjust N:C ratios, and also highlighted the importance of mitochondria in controlling the C-N balance and amino acid homeostasis.

Another study by Rosche et al. (2002) demonstrated that *StSUT1* under control of the pea vicilin promoter, was functionally overexpressed in storage parenchyma cells of developing pea cotyledons. Heterologous expression of *StSUT1* led to a substantial increase in the potential capacity of storage parenchyma cells to transport sucrose from the seed apoplasm. However, this study did not show any increase in the final seed yield or any detectable change in final dry weight of seed (Rosche et al., 2002).

To address this perceived imbalance of C and N supply to the seed, Plant & Food developed pea plants expressing both an amino acid permease and a sucrose transporter. Several double transgenic lines showed enhanced yield. In this thesis, gene expression was first assessed during seed development in the two different cultivars of *Pisum sativum*: a process pea (Bolero) noted for its high sucrose content at time of harvest of the immature seeds, and a field pea (Bohatyr) with its high starch content at the time of harvest of mature seed. Gene

expression of key nutrient transporter genes and other selected genes isolated were monitored throughout seed development to provide the baseline for the transgenic analysis. The second phase of the project was to analyse different transgenic lines of process and field peas, to assess the impact on expression of genes during seed development, especially of genes considered likely to have played a key role in the increased yield and seed number of the transgenic plants that expressed the *SUT* and *AAP* genes. Additionally, the impact of the transgenes on leaves subtending developing pods was studied.

The transgenic plants were the previously developed lines of process and field pea, over-expressing a pea sucrose transporter (*PsSUT1*) and/or amino acid permease (*PsAAP1[3]*) under the control of either the 35SCaMV promoter or a tissue specific promoter (*AtAAP1*). The *PsAAP1[3]* transporter had been chosen because it was able to recognize many of the amino acids present in the pea leaf apoplasm (Zhang et al., 2010). The *AtAAP1* promoter turned out not to be seed-specific in the pea plant but to express in the phloem and also the cotyledon (Tan et al., 2010; Zhang et al. 2015). The 35SCaMV promoter was expected to be constitutively expressed. The effect of the transgenes on expression of *SUT1* and *AAP1[3]* family members and on *SWEET*, *cwINV*, *IPT* and *CKX* gene family members was examined. These gene family members were selected in this study as they play pivotal roles in nutrient transport, cell division and metabolism during seed development.

In this study, the results from the phenotypic analyses showed that there was a significant increase in yield in two double homozygote seed lines, which contained both the transgenes. Data also showed that there was an increase in the seed number which included increase in seeds per pod and pods per plant, although there was not much increase in the seed weight. More importantly, there was no decrease in seed weight (Table 1.1). Another finding was that there was no significant change in seed components, except for a greater amount of starch in the dried process peas (Jan E Grant, personnel communication). The yield increase could be a result of over-expression of the transporter genes during the early stages of seed development and also at the other exporting regions such as source leaves, and in seed coats.

During seed development, photoassimilates are transported to the sink tissues from source leaves. Transporter genes, especially amino acid transporters and sucrose transporters play a crucial role in this. Sucrose transporters are co-localized to plasma membranes of sieve

element/companion cell (se/cc) complexes with H<sup>+</sup>-ATPases and also in plasma membranes of cotyledon epidermal and sub-epidermal transfer cells on the seed coat.

The data shows that the expression of nutrient transporter genes and other gene family members was up-regulated in the young source leaves. The results also show that *PsSUT1a* and *PsSUT1b* were greatly expressed in the leaves which agrees with the findings that *PsSUT1* is expressed in non-seed tissues, which suggests a wider role of this gene in sucrose transport and phloem loading in leaves (Tegeder et al., 1999). *PsSUT1a* is the transgene in this study and showed significantly higher expression in the younger source leaves of transgenic lines (Fig 5.3A). This can be due to the pull from sink leading to greater photosynthetic activity in the young source leaves, and the nutrient transporter genes are highly active in phloem loading of photoassimilate out of these leaves towards the sink tissues.

During the early stage of legume seed development, the maternal seed coat modifies and controls the nutrient supply to the embryo, thus regulating the cell division phase (Weber et al., 1997). Nutrient transporter genes play an important role in unloading the photoassimilates in these regions. It is evident from the data that the nutrient transporter genes were expressed greatly in seed coats. This means, the maternal seed coat is playing the central role as a sink importing photoassimilates from source leaves, then as a source for the developing cotyledons. Another finding from the data is that most of the gene family members showed elevated expression in seed coats, at 12- 14 and 16- 20 DAF, during which the storage of nutrients is at its maximum, and the seed coat is importing nutrients from the source. Interestingly, in seed coats of double transgenic lines the expression of several different gene families was significantly increased, which indicates that the presence of the transgenes influences the regulation of other gene families, indirectly up-regulating them.

The results show that the *SUT*, *AAP*, *cwINV* and cytokinin regulatory genes are expressed greater in cotyledons in the early stages of seed development than the maturing phase. It means during morphogenesis phase, the cell division is maximum and requires high amount of energy. The sucrose transporters play a crucial role for importing sucrose from the source leaves, for providing energy. Interestingly, *cwINV* gene family members show elevated expression simultaneously which is agreeing with another study by Chourey (1992).

In developing pea cotyledons, sink limitation is imposed by processes responsible for starch and storage protein accumulation (Wang, 1993). Imported sucrose and amino acids contribute C and N skeletons for the biosynthesis of these storage compounds (Patrick and Offler, 2001). Sucrose, released from seed coats, is taken up from the seed apoplasm by abaxial epidermal transfer cells, sub-epidermal cells, and one or two outer layers of underlying storage parenchyma cells of pea cotyledons by sucrose transporters (Tegeder et al., 1999, Rosche et al., 2002).

The expression of *PsSUT* genes varied between tissue types and developmental stages. Expression of both *SUT1a* and *SUT1b* was generally greater in pod walls than in seed tissues which is partially supported by the finding of Tegeder et al. (1999) that the signal strength of *PsSUT1* transcripts was weakest in seed coat tissues (Tegeder et al., 1999). On the other hand, it was reported that *Phaseolus vulgaris SUT1* (which has very close sequence homology to the *PsSUT1b*) showed very strong seed specificity, with high expression in cotyledons of developing bean seeds (Zhou et al., 2007). This difference in expression pattern between *PvSUT1* and *PsSUT1b* may suggest that these two species have developed a distinct mode of regulation. It was shown in the data that the sucrose transporter genes are up-regulated at the similar stages when cell division is at its maximum and requiring energy. Therefore, in this case sucrose provides the energy for cell proliferation which is why the *SUT* genes are up-regulated.

Evidence have shown that the identified AAP gene family members are classified into four main clusters and have different functions (Tegeder and Ward, 2012). The isolated PsAAP gene family members studied in this experiment come under Cluster 1, 3A and 4B. Members of Cluster 3A are essential for phloem loading along the transport path (Zhang et al., 2010, Hirner et al., 1998) and are involved in amino acid import into the sieve element/ companion cells complex in legumes (Tegeder and Ward, 2012). Members of Cluster 4B play main role in seed loading, rather than phloem loading of amino acids (Sanders et al., 2009).

Tegeder et al. (2000) found that *AAP1[3]* and *AAP2* gene family members were expressed throughout the Bohatyr plants (field peas), but the transcript levels of *AAP1[3]* were highest

in source leaves and pod walls compared to stems, roots, flowers and seeds, whereas *AAP2* transcript levels were expressed in all organs almost in same amount (Tegeder et al., 2000). The expression of *AAP1/3* was strongest during the seed filling phase, while *AAP2* expression was detected during the early stages of seed coat development. Thus, the expression pattern of *PsAAP3a* in the present study is similar to that observed in the experiments carried out by Tegeder *et al.*, i.e. high *AAP1/3* expression in pod walls. However, this gene family member showed strong expression during later stages of seed development as well.

A number of transgenic studies have also strengthened a correlation between altered cell wall invertase activity and the modification of sink strength in transgenic plants. Transgenic over-expression of cell wall invertase in potato (Sonnewald et al., 1997) and its antisense repression in carrot (Tang et al., 1999) have confirmed that cell wall invertases play crucial role in early differentiation and development of sink organs. Cytokinins have been shown to stimulate unloading of photo-assimilates from excised bean seed coats (Clifford et al., 1986). Moreover, in another study, it was also shown that cell wall invertase transcript levels were highly up-regulated by physiological concentrations of cytokinins (Roitsch and Ehneß, 2000), and the similarity in expression timing and localization of cwINV (Chourey, 1992) and *ZmIPT2* in maize kernels was noticeable.

As suggested in the literature for *B. napus* (e.g.(Song et al., 2015), the isolated *PsINV* gene family members exhibited higher expression during early stages of seed development and also in the seed coats. It was observed in several plant species that an increase in invertase activity occurs in response to hormones such as gibberellins (Wu L, 1993) or cytokinins (Ehneß and Roitsch, 1997). It was not clear whether these effects are due to direct regulation of invertase genes by plant hormones or via stimulated cell proliferation creating new sinks for sucrose.

The key role of *SWEET* gene family members in phloem loading was supported by an earlier study in mutant plants (Chen et al., 2012b). These findings shed light on a significant breakthrough in the concept of phloem transport and the regulatory mechanism of sucrose transport from source leaves to sink tissues. *AtSWEET11* and *12* are highly expressed in leaves and were found to be co-expressed with genes involved in sucrose biosynthesis and phloem loading (e.g., sucrose phosphate synthase, *SUC2*). It was shown in studies that

*AtSWEET10*, *13*, *14* and *15* function as sucrose transporters and also, *AtSWEET13* was expressed at low levels in leaves (Chen et al., 2012b). Another study revealed that *AtSWEET17* was expressed in the parenchyma and the vascular tissue, and functioned as a vacuolar fructose transporter, exporting fructose out of vacuoles and controlling fructose content in leaves (Chardon et al., 2013). It is clear from the data that the transgenic plants showed greater expression for *PsSWEET* gene family members, compared with WT plants. It is evident from literature that *AtSWEET11* and *12* are highly expressed in leaves (Chen et al., 2012a) which supports the result, i.e., high expression of *PsSWEET13a* in young source leaves, which indicates that the stronger sink activity is enhancing uploading from source leaves

Evidence showed that *CKX* gene family members could be functionally differentiated by their spatial and temporal expression patterns (Werner et al., 2001, Werner et al., 2003). Also, it was found that *OsCKX2* was expressed in leaves, inflorescence meristems and flowers (Ashikari et al., 2005a). In *Arabidopsis*, the *IPT* genes are expressed in several tissues, including roots, leaves, stems, flowers and siliques (Miyawaki et al., 2004). Evidence revealed that *AtIPT3* is expressed in phloem tissue in rosette leaves. *AtIPT5* was expressed in lateral roots, upper parts of young inflorescence and fruit abscission zones (Takei et al., 2004, Sakakibara et al., 2006). In this study, the results showed that the isolated *PsIPT4* was expressed during the later stages of seed development, which can be related to a result from Emery et al. (2000), which stated that dihydro cytokinin forms were detected not only during early stages of seed development, but a significant level of cytokinin was found throughout seed development.

As shown in the data, cytokinin biosynthesis genes were not highly expressed in leaves, which means cytokinin biosynthesis is very low in leaves. Evidence shows that there is a very limited synthesis of cytokinin in leaves (Miyawaki et al., 2004). But expression of these genes were greater in the double transgenics, especially in field pea lines, compared with the WT. However, strong expression of *PsCKX* genes, especially *PsCKX1* could be seen in the data. This result agrees with the finding that cytokinin is supplied to leaves from roots, in which they suggested that roots were the major sites of tZ production and that cytokinin was transported to shoot (Hirose et al., 2008, Takei et al., 2004). Also, it was found that *OsCKX2*



was expressed in leaves, inflorescence meristems and flowers (Ashikari et al., 2005b). The CKX gene family members have been shown to express in different plant tissues and to play essential roles in controlling cytokinin levels during plant growth and development (Schmülling et al., 2003).

In summary, this study reveals that there is an overall increase in the seed yield in double transgenic lines, without changing the seed components, and by maintaining the same seed size. It is evident from the data that yield increase could be a result of enhancing both sucrose and amino acid import to seed, along with enhanced cytokinin and cell invertase activity during early stages of development. And also, the strong impact of young source leaves and seed coats on the nutrient flow to the developing sink, which opens up a very interesting area of source- sink interaction during different stages of seed development. It is also evident from the data that the double transgenes have much wider effect on whole plant rather than just at the sites of expression.

# Appendices

## A. Chapter 2

### Recipes

#### 2.1 TPS (Template Preparing Solution) buffer

(for 10 ml)

6.8 ml H<sub>2</sub>O

1 ml 1M Tris-HCl (pH 9.5)

2 ml 2.5 M KCl

200 µl 0.5 M EDTA

#### 2.2 25X TAE buffer

121g Tris base

28.5ml glacial acetic acid

9.3g EDTA

Made up to 1L with nanopure water

#### 2.3 TE Buffer

10 ml 1 M Tris (pH 8)

2 ml 0.5 M EDTA (pH 8)

Made up to 1 L with nanopure water

#### 2.4 1 M Tris Stock

121 g Tris base

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

## **2.5 0.5 M EDTA stock**

146 g EDTA

Made up to 1 L with nanopure water, Adjust pH to 8.0 with HCL

## **2.6 6X Agarose gel loading dye**

60mM EDTA

10mM Tris-HCl (pH 7.6)

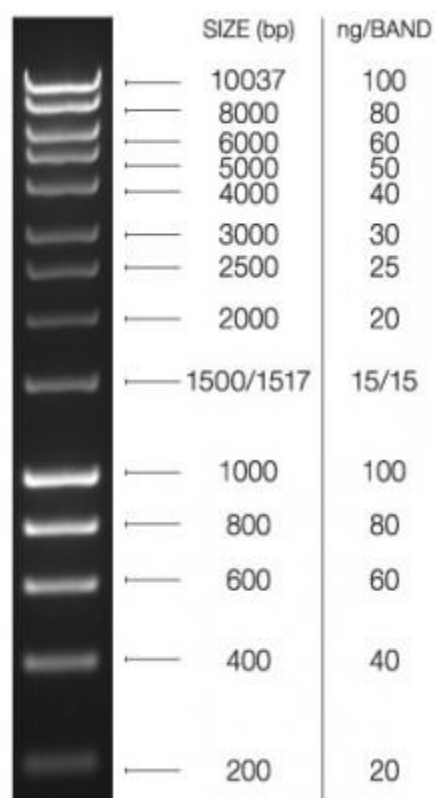
0.03% Xylene cyanol FF

0.03% Bromophenol blue

60% glycerol

## **2.7 Bioline HyperLadder™**

An agarose gel run with 5 µl of HyperLadder™ 1.



## 2.8 Primer annealing mix

1 – 4  $\mu$ l RNA (depending on purity and concentration)

1  $\mu$ l pDN6 random primers (100 pmoles)

1  $\mu$ l oligo (DT) primers (50 pmoles)

0.5  $\mu$ l 25x RNasecure

Made up to 10  $\mu$ l using DEPC H<sub>2</sub>O

## 2.9 Reverse Transcriptase mix

4  $\mu$ l RT buffer (5x)

1  $\mu$ l dNTPs (20 mM)

2 µl DTT

2 µl DEPC H<sub>2</sub>O

1 µl Expand Reverse Transcriptase enzyme (50 U/µl)

### **2.10 2x SYBR green RT-qPCR reaction buffer**

15 µl SYBR green (200x)

930 µl qPCR buffer (2x)

40 µl dNTPs (20 mM)

15 µl Bioline taq polymerase

### **2.11 200x SYBR green**

2 µl 10,000x SYBR Green 1 (Invitrogen)

998 µl DMSO

### **2.12 2x RT-qPCR buffer**

227 mg Trehalose

340 µl H<sub>2</sub>O

200 µl qPCR buffer (-Mg) (10x)

160 µl MgCl<sub>2</sub> (50 mM)

80 µl DMSO

30 µl 10% Triton-X 10

### **2.13 MOPS buffer (for starch assay)**

1. Combine

1.155g MOPS salt

90 ml H<sub>2</sub>O

2. Adjust pH to 7.0 with NaOH

3. Then add and dissolve

74 mg Calcium chloride dihydrate

4. Make up to 100 ml

### **2.14 Sodium acetate buffer (200mM, pH 4.5) (for starch assay)**

1. Combine

11.8 ml glacial acetic acid

900 ml water

2. Adjust pH to 4.5 with 1M sodium hydroxide

3. Make up to 1 litre

### **2.15 Solutions for sucrose assay**

#### **2.15 a) Solution 1**

30 mg NAD

60 mg ATP

100 µl 1M MgCl<sub>2</sub>

13 ml 0.2M triethanolamine buffer pH 7.6 (TAE)

13 ml H<sub>2</sub>O

#### **2.15 b) B-Fructosidase**

Stock solution

2 mg B-Fructosidase

700 µl citrate buffer

Working solution dilute 1: 10

For 100 samples

100 µl      stock solution

900 µl      citrate buffer

### **2.15 c) Citrate buffer 0.1M**

Mix

44.5 ml    0.1M citric acid

55.5 ml    0.1M trisodium citrate

Adjust pH to 4.6

### **2.15 d) HK/G6PDH**

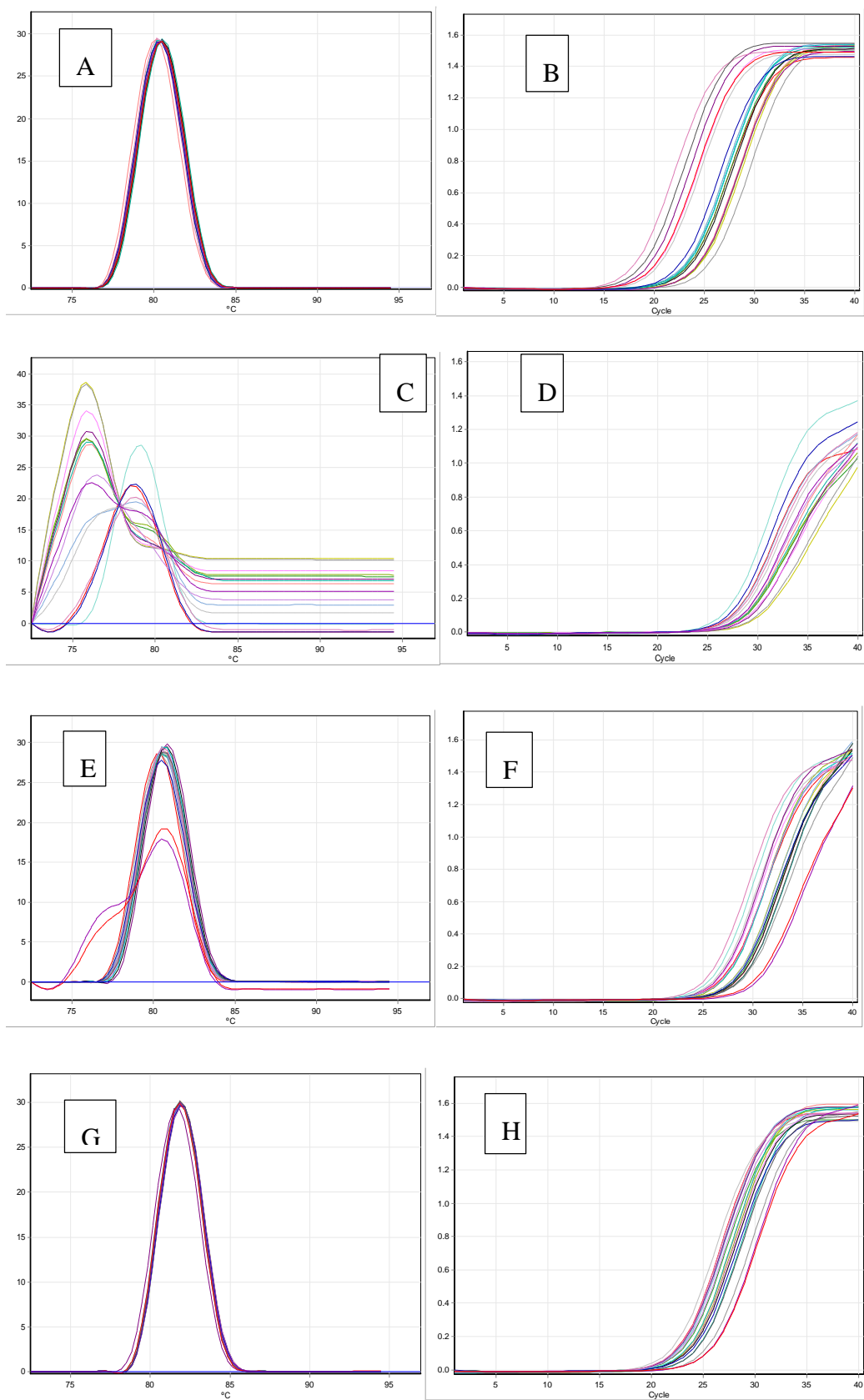
260 µl      suspension (Roche 10737275001)

800 µl      solution 1

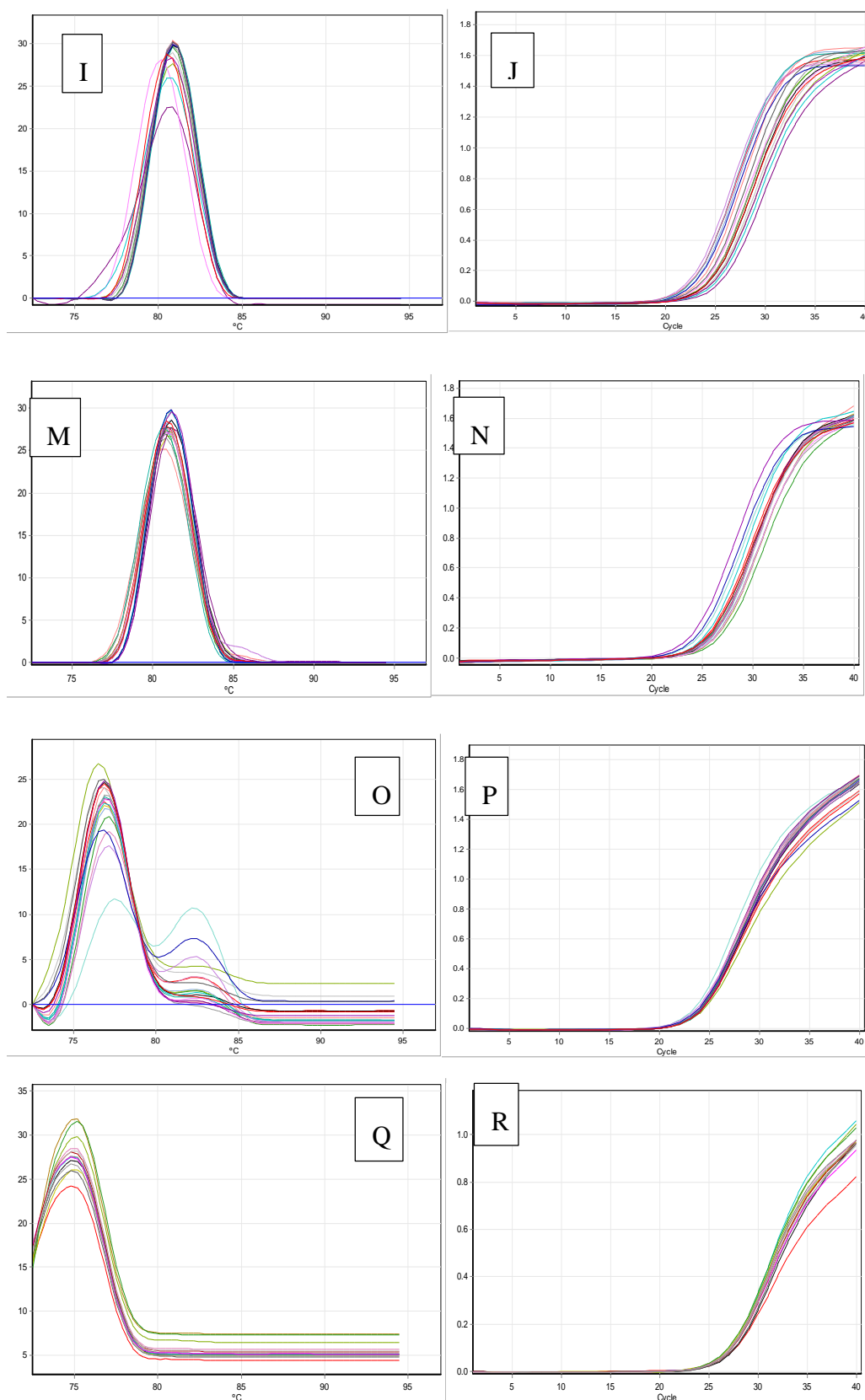
## **B. Chapter 3**

### **3.1 Melting and Amplification curves**

#### **3.1.1 Melting and Amplification curves of *PsIPT* and *PsCKX***



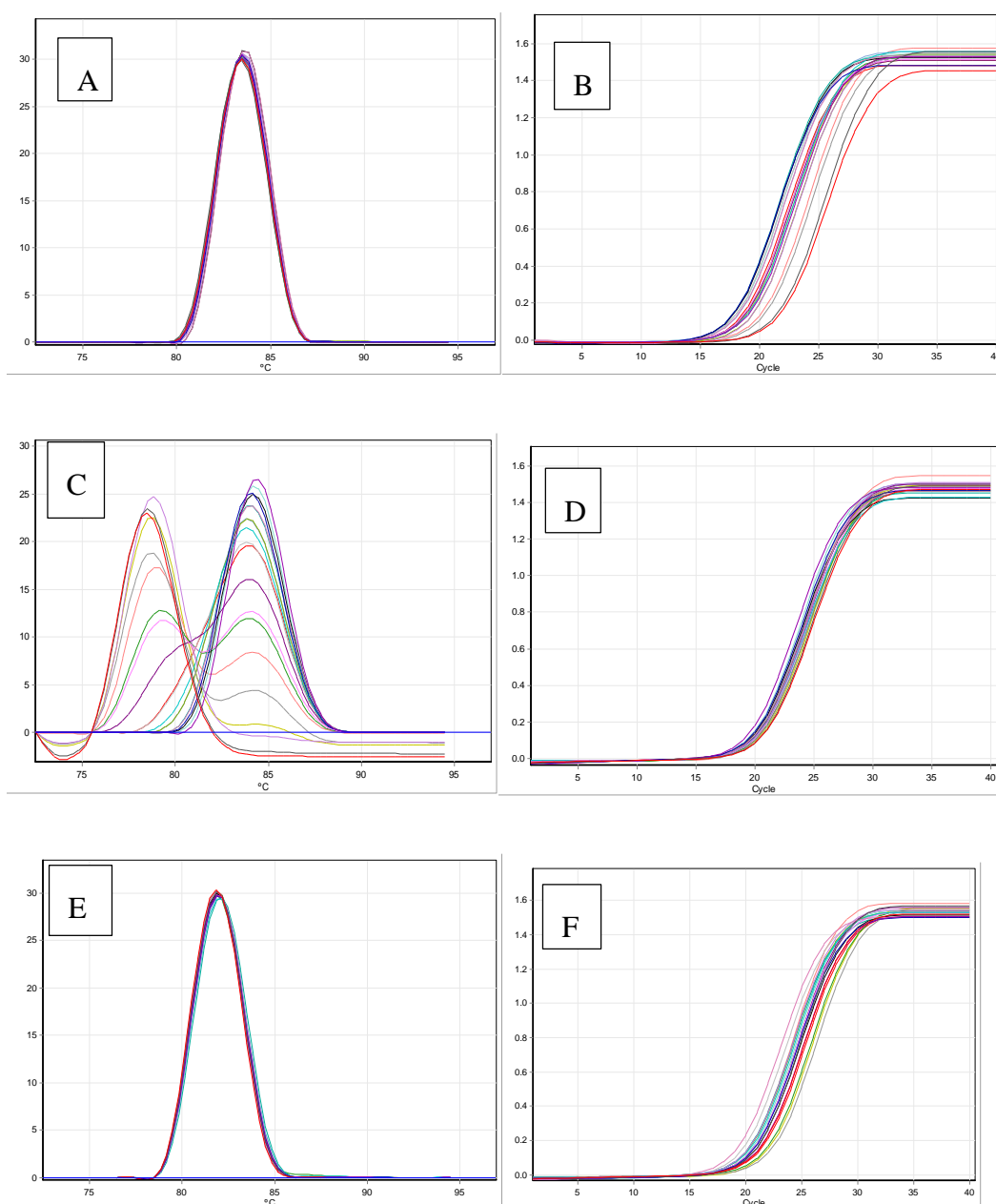


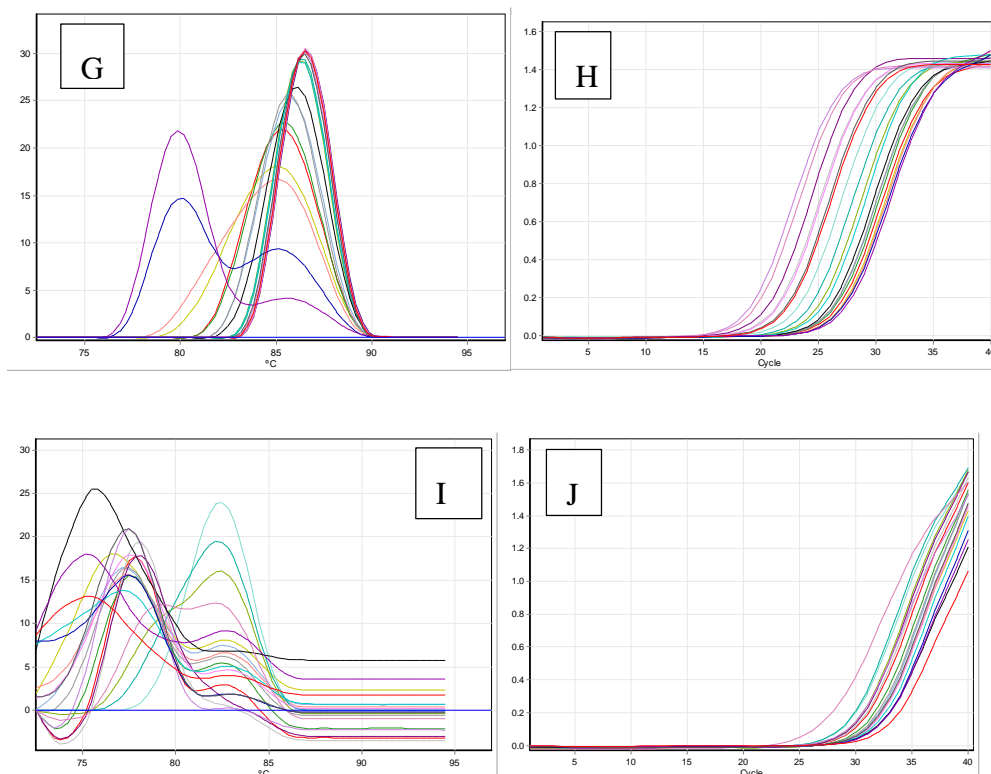


**Figure 3.1.1: RT-qPCR profiles of *PsCKX* and *PsIPT* genes.** A,B- melting and amplification curves for *PsCKX1*. C,D- melting and amplification curves for *PsCKX2*. E,F- melting and amplification curves for *PsCKX4*. G,H- melting and amplification curves for *PsCKX5*. I,J- melting and amplification curves for *PsCKX6*. K,L- melting and amplification

curves for *PsCKX3b*. M,N- melting and amplification curves for *PsIPT2*. O,P- melting and amplification curves for *PsIPT4*. Q,R- melting and amplification curves for *PsIPT1*.

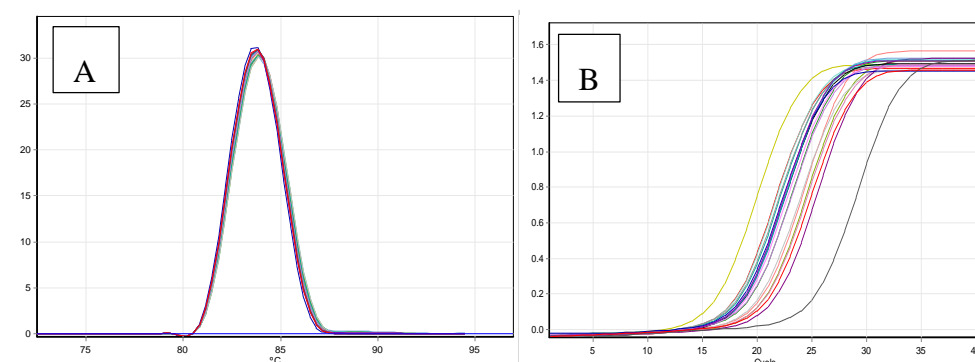
### 3.1.2 Melting and Amplification curves of *PsSUT*

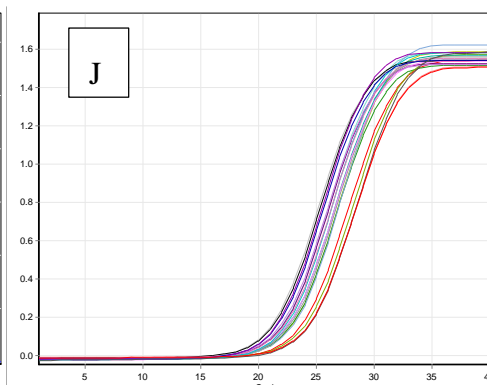
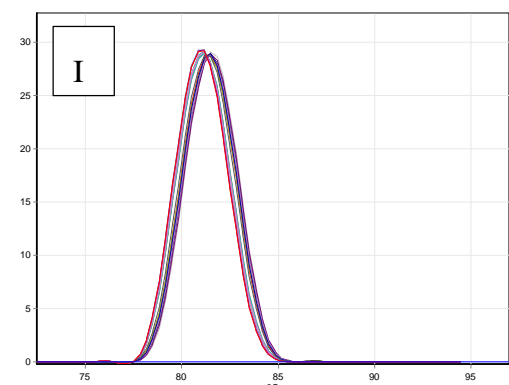
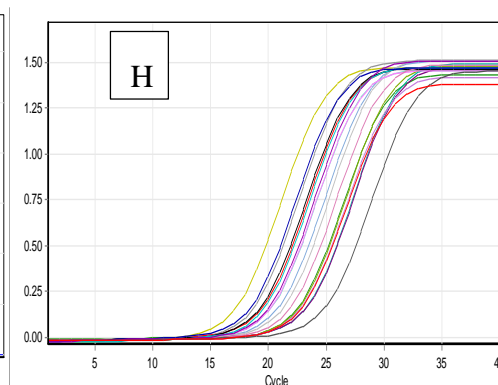
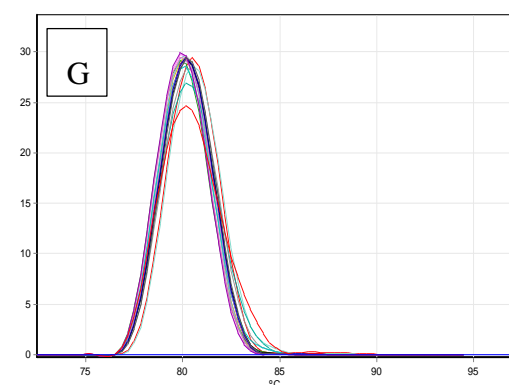
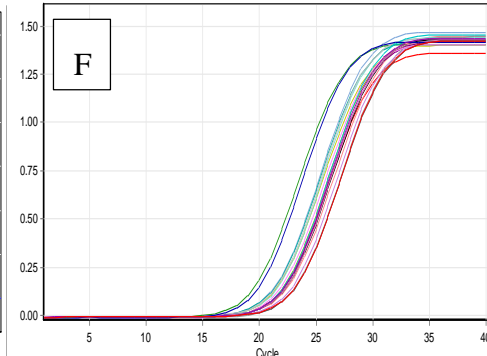
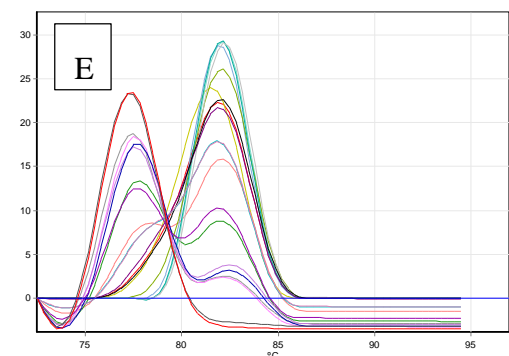
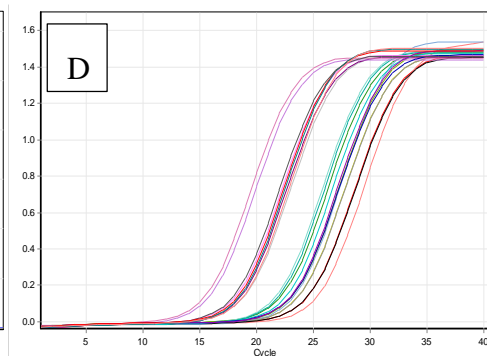
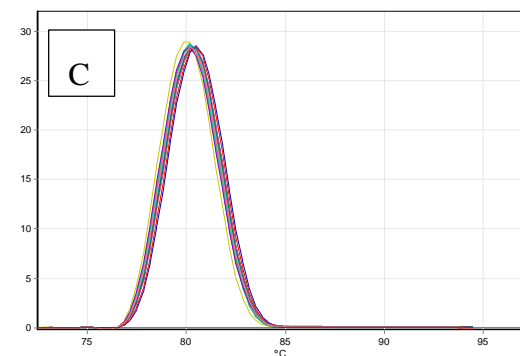


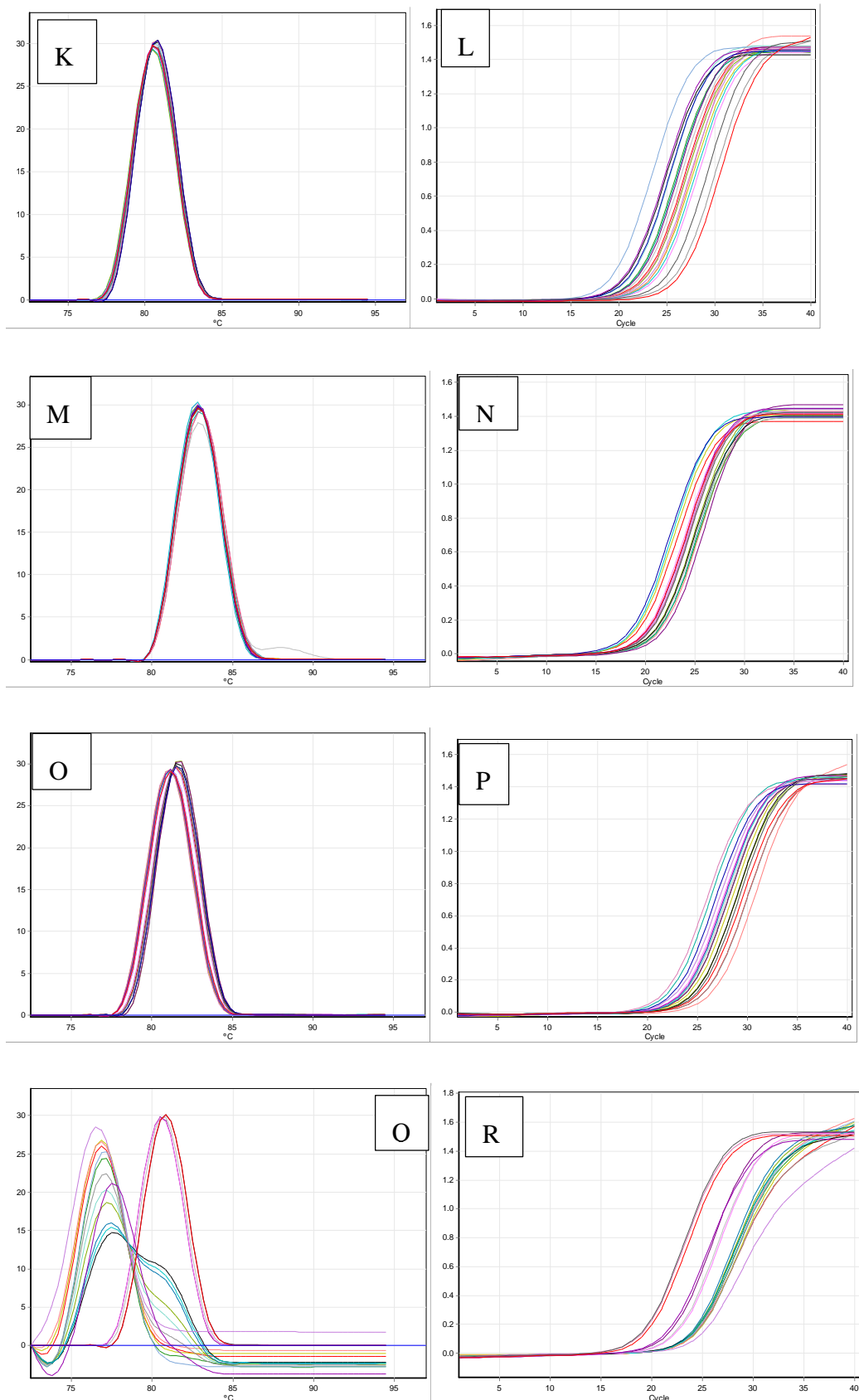


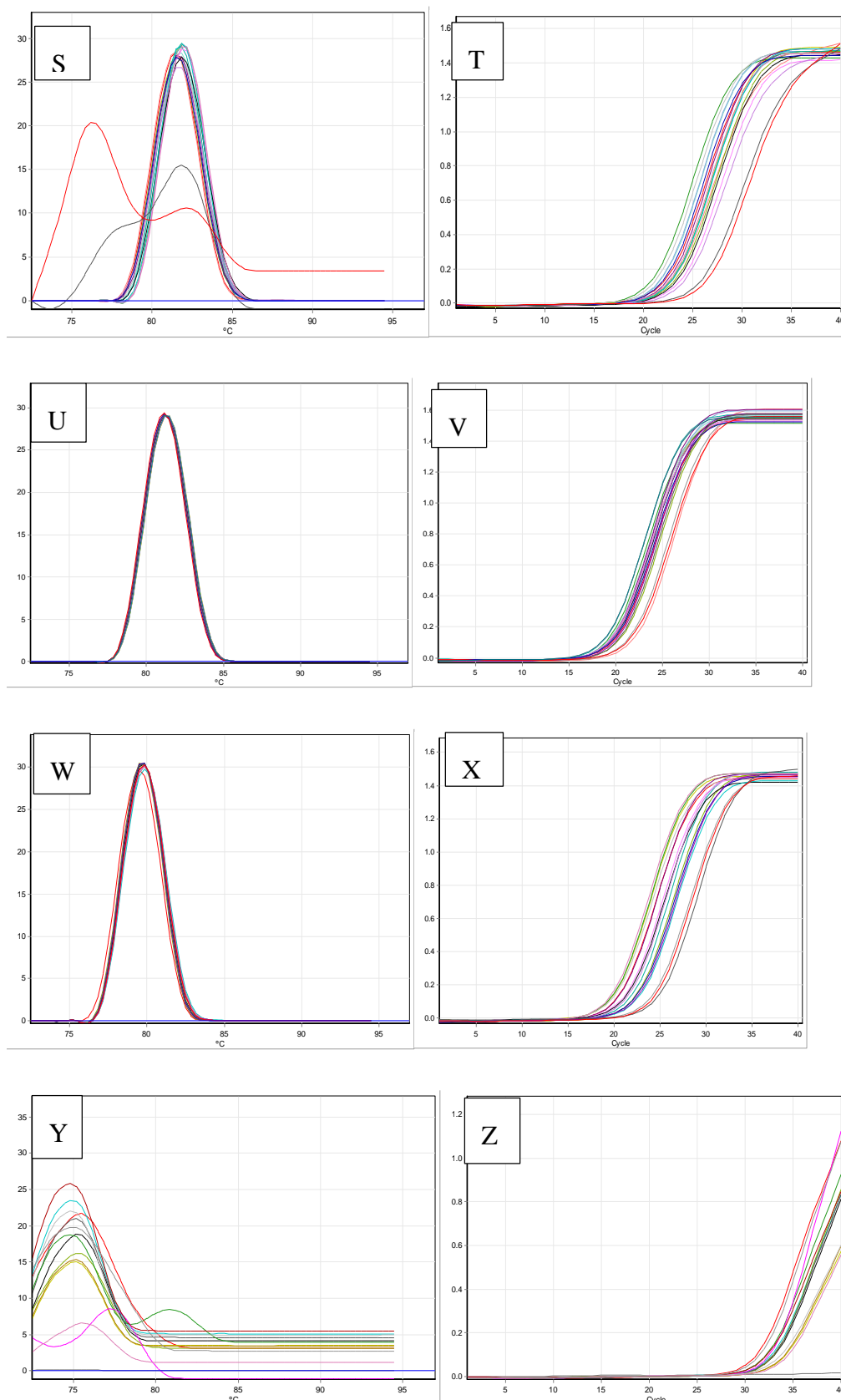
**Figure 3.1.2: RT-qPCR profiles of *PsSUT* genes.** A,B- melting and amplification curves for *PsSUT1a*. C,D- melting and amplification curves for *PsSUT1b*. E,F- melting and amplification curves for *PsSUT2*. G,H- melting and amplification curves for *PsSUF1*. I,J- melting and amplification curves for *PsSUT10*.

### 3.1.3 Melting and Amplification curves of *PsAAP*





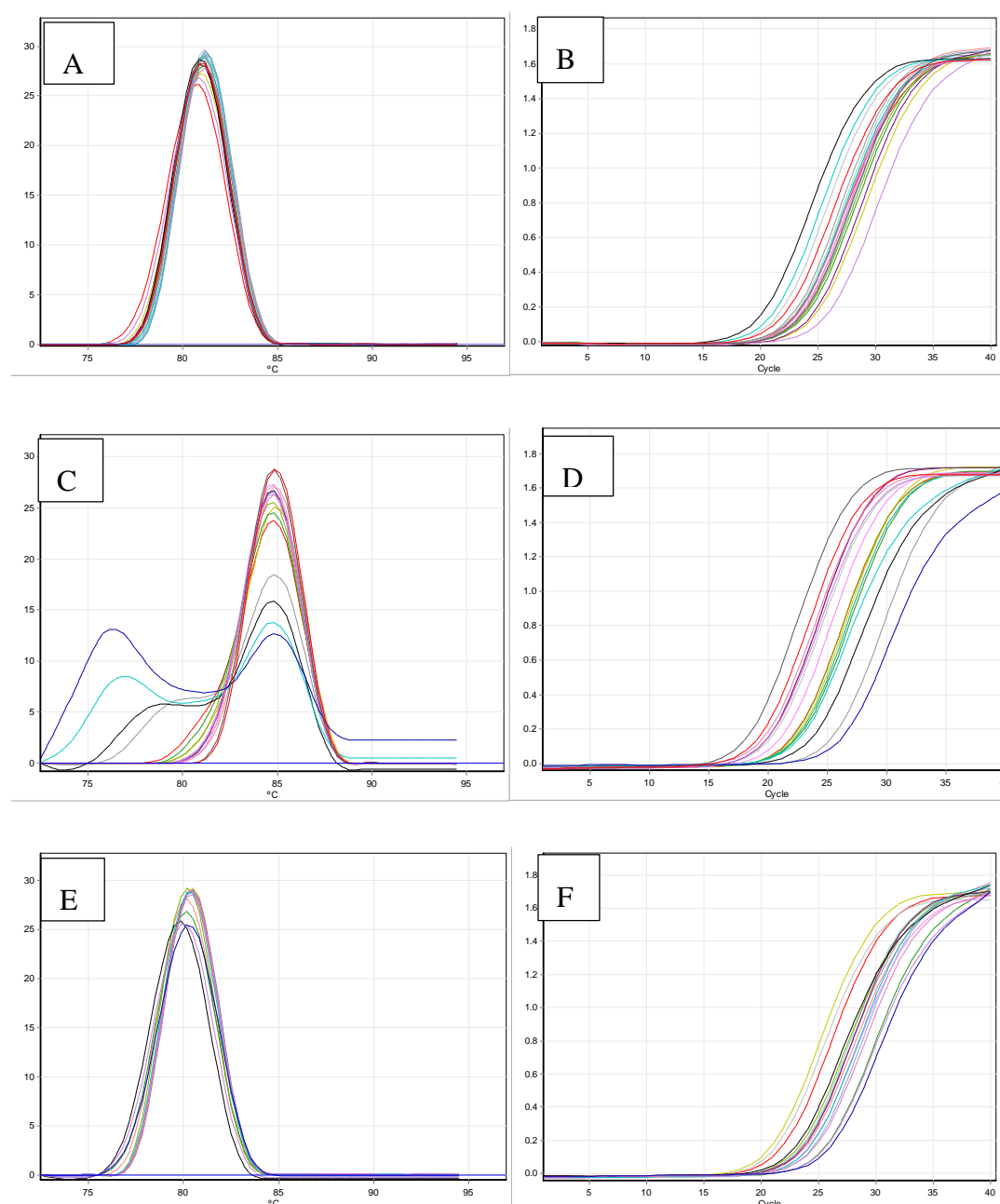


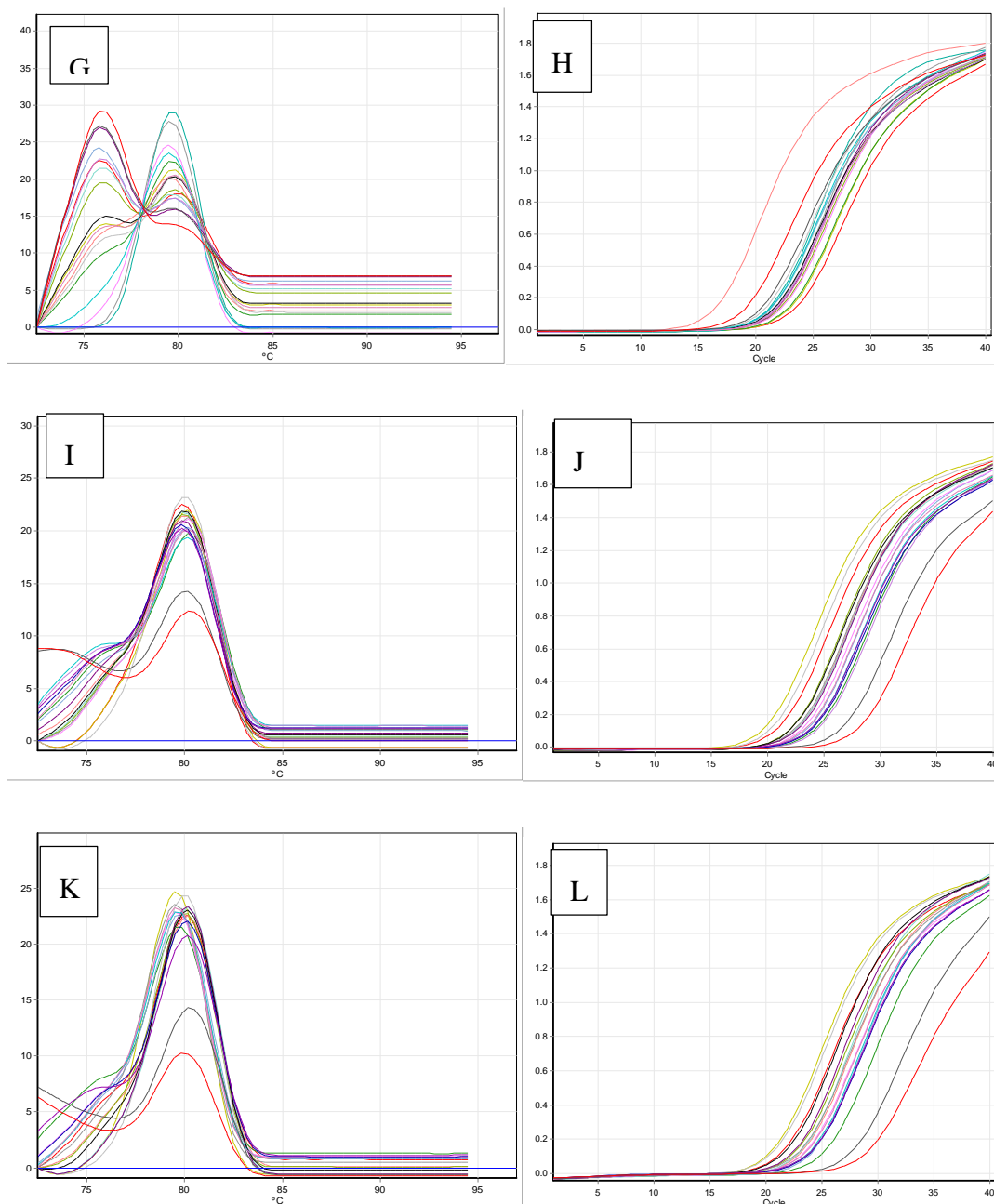


**Figure 3.1.3: RT-qPCR profiles of PsAAP genes.** A,B- melting and amplification curves for PsAAP3b. C,D-melting and amplification curves for PsAAP1. E,F- melting and amplification curves for PsAAP2e. G,H- melting and amplification curves for PsAAP6d. I,J-

*melting and amplification curves for PsAAP7b. K,L- melting and amplification curves for PsAAP3a. M,N- melting and amplification curves for PsAAP2a. O,P- melting and amplification curves for PsAAP7e. Q,R- melting and amplification curves for PsAAP6c. S,T- melting and amplification curves for PsAAP2d. UV- melting and amplification curves for PsAAP2b. W,X- melting and amplification curves for PsAAP7c. Y,Z- melting and amplification curves for PsAAP8*

### 3.1.4 Melting and Amplification curves of *PsINV*

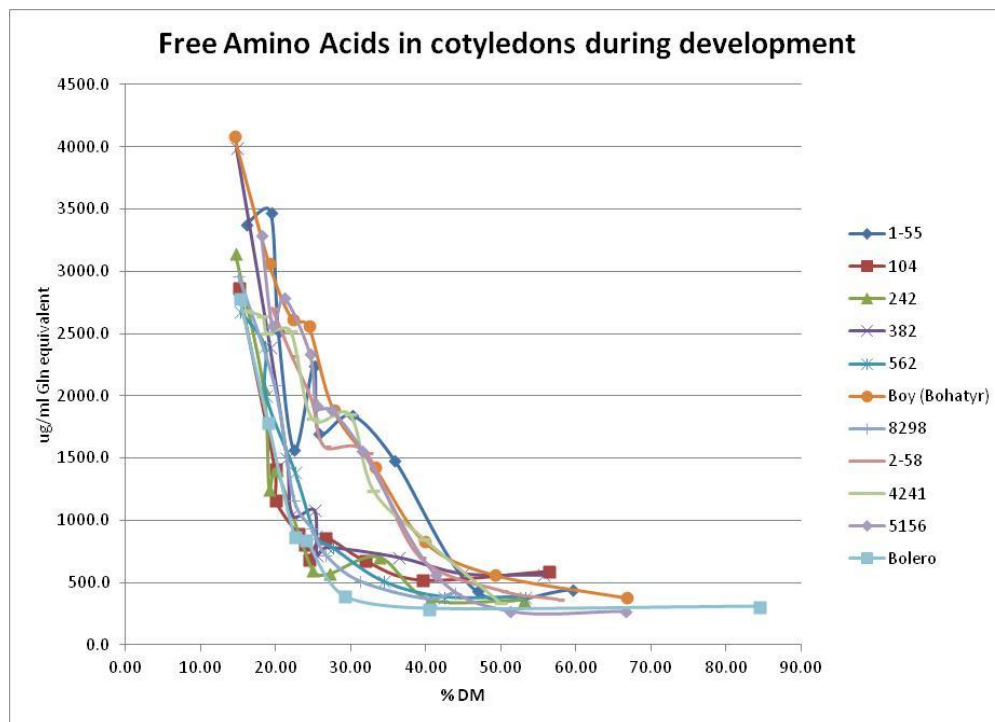


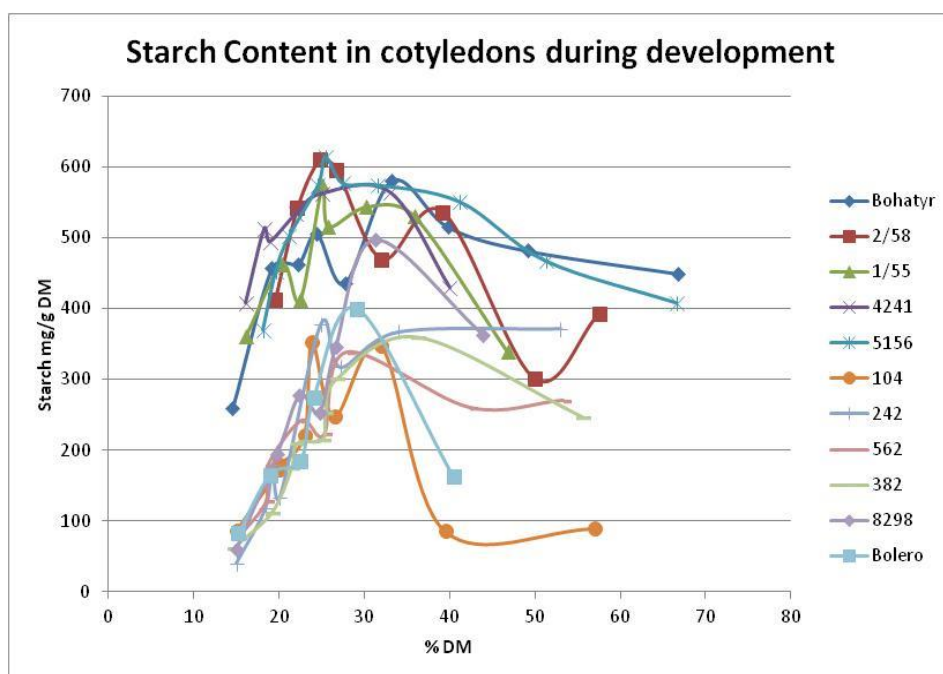
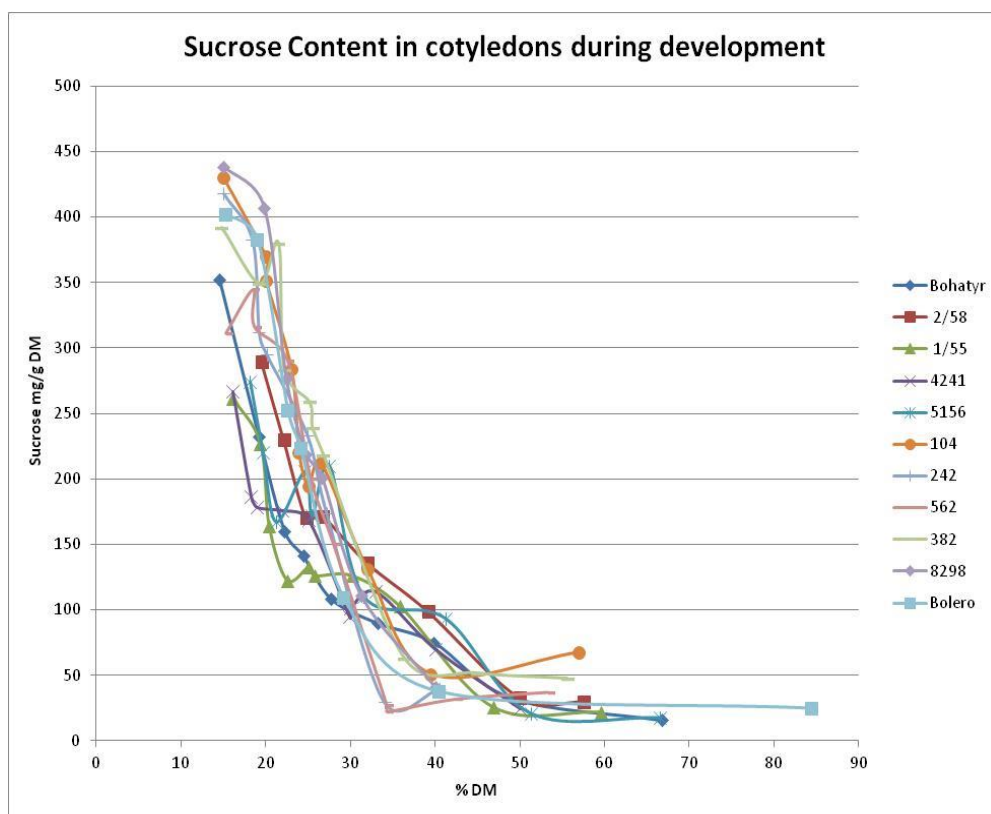


**Figure 3.1.4: RT-qPCR profiles of *PsINV* gene.** A,B- melting and amplification curves for *PsINV*448. C,D- melting and amplification curves for *PsINV*240. E,F- melting and amplification curves for *PsINV*285. G,H- melting and amplification curves for *PsINV*320. I,J- melting and amplification curves for *PsINV* 415. K,L- melting and amplification curves for *PsINV* 681.



### 3.2 Starch, sugar, and amino acid analyses data, performed by Plant & Food personnel





**Figure 3.1.5: Sucrose, starch and amino acid content in the developing cotyledons.** A is the amino acid content in  $\mu\text{g/ml}$  of glutamine equivalent against % dry matter. B and C is sucrose and starch in  $\text{mg/g}$  of dry matter against % dry matter. (The analyses were completed by Plant& Food personnel. Data courtesy of Jan Grant)

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